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CANCER RESEARCH

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Numerical Proportions of Different Cells in Mouse Epidermis during Early Methylcholanthrene Carcinogenesis*

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It is important to make every effort to interpret correctly the changes in the properties of the epidermis of mice during carcinogenesis induced by cutaneous applications of 0.6 per cent methylcholanthrene in benzene. These observations, recently summarized (2, 3), reveal what appear to be remarkable increases in some chemical constituents, decreases in others, and unexpected consistencies in others. Obviously, alterations in the relative numerical proportions of cells, themselves of different chemical composition, may be factors worthy of consideration. If, for instance, the spinous cells have a particularly low calcium content and they increased in number relative to the basal and granular cells in epidermal carcinogenesis, this would explain the decrease in calcium of whole epidermis reported. If, for example, the granular cells maintain the same lipid content but their number relative to the other cells decreases, this would explain the decrease in total lipids of whole epidermis. In both of these cases the changes in composition of whole epidermis might take place without alteration in the composition of the cells.

An investigation of the alterations in the epidermal cellular population, which is partly or wholly replaced during the period of carcinogenesis, is evidently one of the necessary checks on the findings. A careful study of this epidermal population of mice has indeed already been made by Glücksmann (5), but the carcinogen employed by him was benzpyrene. The chemical changes brought about by this agent may be similar to those induced by methylcholanthrene; but evidence is lacking that this is so, because the responding epidermis in his series was not subjected to the same methods of chemical analysis as in the series under review. Consequently, the necessity remains of investigating the cellular population changes in mice of the same inbred strain and subjected to the same carcinogen used in the experiments to be checked. Moreover, it has seemed desirable to make use of rather different technics from those of Glücksmann.

Three so-called "types" of cells are ordinarily recognized in mouse epidermis when it becomes hyperplastic soon after the methylcholanthrene is first applied. These are basal cells, spinous cells, and granular cells. They are not, strictly speaking, of different types, for all three are epidermal cells in the same line of differentiation in the proximodistal sequence of epidermal replacement and of the same ectodermal origin. Their individual lives are terminated by death, or by mitosis. Degeneration leading to cell death occurs in small minorities of basal and of spinous cells. All the granular cells can be regarded as degenerating or dying, for they are senescent and obviously near the end of their lives. Mitosis is evident among basal and spinous cells. But the frequency of mitosis in the epidermal population is small. According to Cooper and Reller (1), in the ear epidermis of Strain A mice it is normally only 0.11 per cent. It is for our purpose not worth while to consider the possibly existing melanoblasts (dendritic cells) or their precursors, for it is unlikely that either constitutes a

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Data from a dissertation submitted to Washington University in partial fulfillment of requirements for the Ph.D. degree.

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fraction of the population large enough in these albino mice to influence materially, through variations, the results of chemical analysis of whole epidermis. For the same reason, occasional invading cells of mesenchymatous origin (lymphocytes, leukocytes, etc.) can be disregarded. The main issue is to determine the relative numerical proportions of basal, spinous, and granular cells during epidermal carcinogenesis, for these make up the bulk of the epidermis analyzed.

METHODS

Fifty-four female mice of a Swiss albino strain obtained from Albino Farms, Red Bank, New Jersey, were employed. These were approximately 76 days old, and their average weight was 24.5 gm. when the experiments were started.

The first group, consisting of three mice, provided unshaved skin in which to establish the normal cellular population antedating any unusual or abnormally imposed conditions.

The second group, consisting of seventeen mice, simply had the hair shaved from an area extending from the neck to the tail to flanks on both sides. In shaving them, and the mice of the third and fourth groups, care was taken to reduce mechanical injury of the epidermis to a minimum. A safety razor was used with water but no soap. As nearly as possible exactly the same procedure was employed uniformly for all.

The third group, made up of seventeen mice, was shaved, and the shaved areas were then uniformly treated with benzene delivered by one stroke of a No. 4 camel's hair brush in a cephalocaudal direction. The number of applications is shown in Chart 1.

The fourth group, consisting likewise of seventeen mice, was shaved, and the shaved areas were then uniformly treated with 0.6 per cent methylcholanthrene delivered in the same manner.

Wide skin samples were excised from the control mice and from groups 2, 3, and 4 at the intervals specified in Chart 1. These excisions were performed immediately after the mice were sacrificed and by a standardized technic. The time always chosen was 10 A.M., in order to secure specimens in the same stage of the 24-hour mitotic frequency rhythm (1).

The fresh specimens were spread out flat, each on a piece of blotting paper, to prevent curling. They were then immediately plunged, mounted

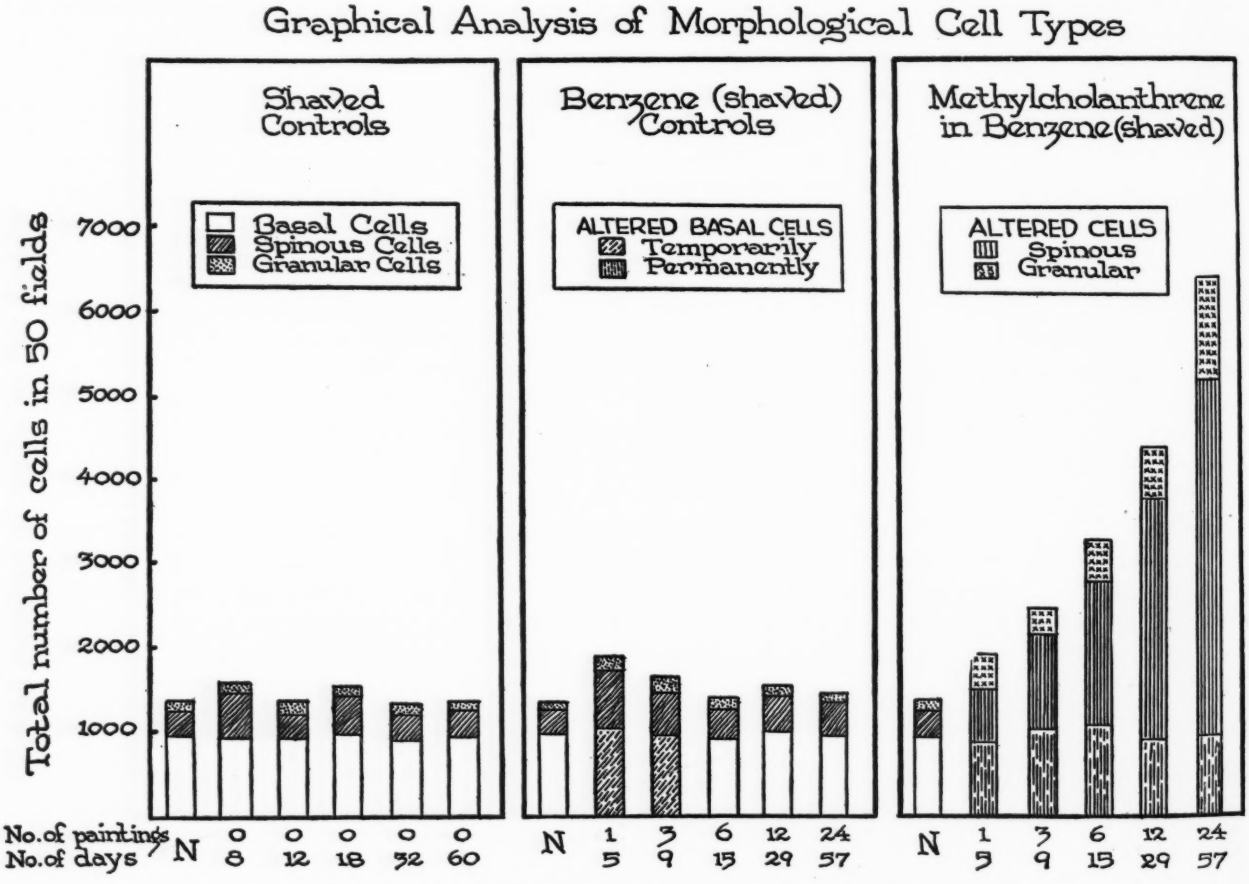


CHART 1.—"N" indicates the cell type proportions of normal, unshaved mouse epidermis

on the blotting papers, into Bouin's fluid. After fixation the edges of the piece of skin were cut down with a razor to an area 1 × 2 cm. Dehydration, clearing, and imbedding in paraffin were uniformly carried out for all. Eight serial longitudinal sections, taken in the mid-line and 5 μ thick, were mounted on each slide. These were routinely stained with Harris' hematoxylin and eosin. In addition, some specimens were colored with the Verhoeff elastic tissue reagents in order more accurately to visualize the granular cells. This tinctorial reaction is perhaps the most effective means of demonstrating keratohyalin granules.

Hair follicles, and the epidermis lining the pits from which the hairs normally protrude, were excluded from the counts. Only portions of vertical sections through flat sheets of epidermis between these pits, oriented as described, were examined in detail.

The counts were all made with the help of 1.8 mm. oil immersion objective and a 10× ocular giving a magnification of 950 diameters. The counting of cells per microscopic field is convenient and not so objectionable as might appear at first sight. When the epidermis is thin, this is viewed as a band of tissue stretching diametrically across the field of vision. The fact that the extremities of this band are slightly convex, so that the amount of tissue included in the center of the band is somewhat greater than that in the upper and lower parts of the band, is not a serious source of error.

The counts were in reality counts of nuclei which were sharply stained and outlined, rather than of cells, for the cell outlines were sometimes indistinguishable, especially in the stratum granulosum. Since detailed search showed that binucleated epidermal cells are of rare occurrence and revealed no multinucleated epidermal cells in the area counted, the assumption was made that these counts of nuclei are substantially equivalent to direct counts of cells.

RESULTS

The accurate identification of basal, spinous, and granular cells posed insurmountable difficulties, because transitions exist between them. The classification employed was therefore arbitrary, as follows:

Basal cells were identified as those in contact with the basement membrane, which partition is presumed to separate, if even inconspicuously, the epidermis from the underlying dermis. The special stain recommended by Gersh and Catchpole (4) was not used to define the position of this membrane, as this seemed unnecessary.

Spinous cells were listed as those located between the basal cells proximally and the granular cells distally. While the majority of them were fully equipped with large or small spines, others had spines only on parts of their surface, and still others no recognizable spines.

Granular cells were identified as possessing more or less granular keratohyalin material in their cytoplasms. But they are simply spinous cells, which in aging had lost their spines and which had been displaced in a distal direction by growth pressure due to cellular multiplication proximal to them. Exactly when in this transition the designation "spinous" should be dropped and "granular" should be substituted is difficult to say. Cells have been listed in these counts as "granular" in which the presence of granules can be detected at a glance and in which the nuclei are fairly prominent.

The same cells further displaced in a distal direction became keratinized, and their nuclei became either flattened or no longer recognizable. Neither these keratinized cells, nor the flattened masses of keratinized material devoid of nuclei, are included in the cell counts. However, in the check that was being attempted of the results of chemical analysis, the fraction of total epidermis made up chiefly of keratinized cellular debris must not be ignored. Every effort has been made to include in the sections all this debris normally existing by very gentle manipulation throughout.

The numerical results have been combined into a single graph, Chart 1, which shows in three sections the effects of shaving, of painting with benzene, and of painting with methylcholanthrene in benzene on the relative proportions of the three cell types. In each section *N* indicates the cell type composition of normal, unshaved epidermis, which amounts to 73 per cent basal, 20 per cent spinous, and 7 per cent granular.

The original enumerations (omitted for the sake of brevity) also gave information on the proportions of those cells of each type which were observed as dividing or degenerating. Thus, of the basal cells of *N* about 3 per cent were classified as in division and about 10 per cent in degeneration; of the spinous cells about 1.5 per cent were degenerating, with none in division; while, of the granular cells, none were in division or degeneration. Where striking deviations subsequently occurred will be noted in the text.

Normal unshaved epidermis.—This is important to us, because we wished to establish the effect of shaving on the epidermis. The epidermal cells are firmly bound together in three layers. By definition, the basal cells constitute the proximal layer, that is, the one nearest to the underlying dermis.

There is an irregular, single, intermediate layer of spinous cells with spines but feebly developed. A distal, single layer of granular cells was observed in all but one of the 50 microscopic fields counted. Immediately distal to the granular cells were several layers of keratinized debris. Loss of some debris is inevitable in making the preparations; but its amount in relation to the amount of cells counted is not of great consequence, because the chemical determinations were not made of hairy epidermis but of normal epidermis that had been shaved.

All the above cells had faintly stained cell membranes without definite spines, and their cytoplasms appeared microscopically homogeneous. The granular cells contained fine, dustlike, keratohyalin granules. The cytoplasm of the spinous cells stained lightly, whereas the cytoplasm of the basal cells colored a little more strongly, and the cytoplasm of the granular cells stained most heavily of the three; but in none of these cell types was the stain very intense. The nuclei of the basal and granular cells were colored to about the same degree, which was somewhat darker than the staining of the spinous cell nuclei (Fig. 1). No abnormal mitoses were observed, but special search was not made for them.

Shaved epidermis.—The effect of a single shaving on the proportions of epidermal cells was studied on groups of three or four mice sacrificed 8, 12, 18, 32, and 60 days thereafter.

Cell membranes were not noticeably altered, except that distinct spines were seen between the spinous cells on the 8th day (Fig. 2). The cytoplasm of the basal cells appeared foamy until the 32nd day, and that of the spinous and granular cells until the 12th day, after which time their appearance returned to normal. The granules of the granular cells were a little larger than normal on the 8th and 12th days. The cytoplasm of the basal cells stained more heavily until the 32nd day, but reached a maximum on the 8th day. The cytoplasm of the spinous and granular cells stained more heavily than normal throughout the entire period of observation. Six to eight layers of keratinized debris were counted over three cell layers of epidermis.

In the basal layer the percentage of dividing cells remained about the same (3 per cent), but the proportion of degenerating cells almost doubled, from 10 to 19, on the 8th day after shaving; however the proportion fell to less than 7 per cent after 32 days.

In the spinous layer the proportion of degenerating cells rose from 1.5 to 14 per cent and subsided to 2 per cent after 32 days.

Shaved, benzene-painted epidermis.—The effect of benzene painting on the proportions of epidermal cells was examined in groups of three or four mice sacrificed after a series of 1, 3, 6, 12, and 24 paintings, on 5, 9, 15, 29, and 57 days after the first treatment, respectively.

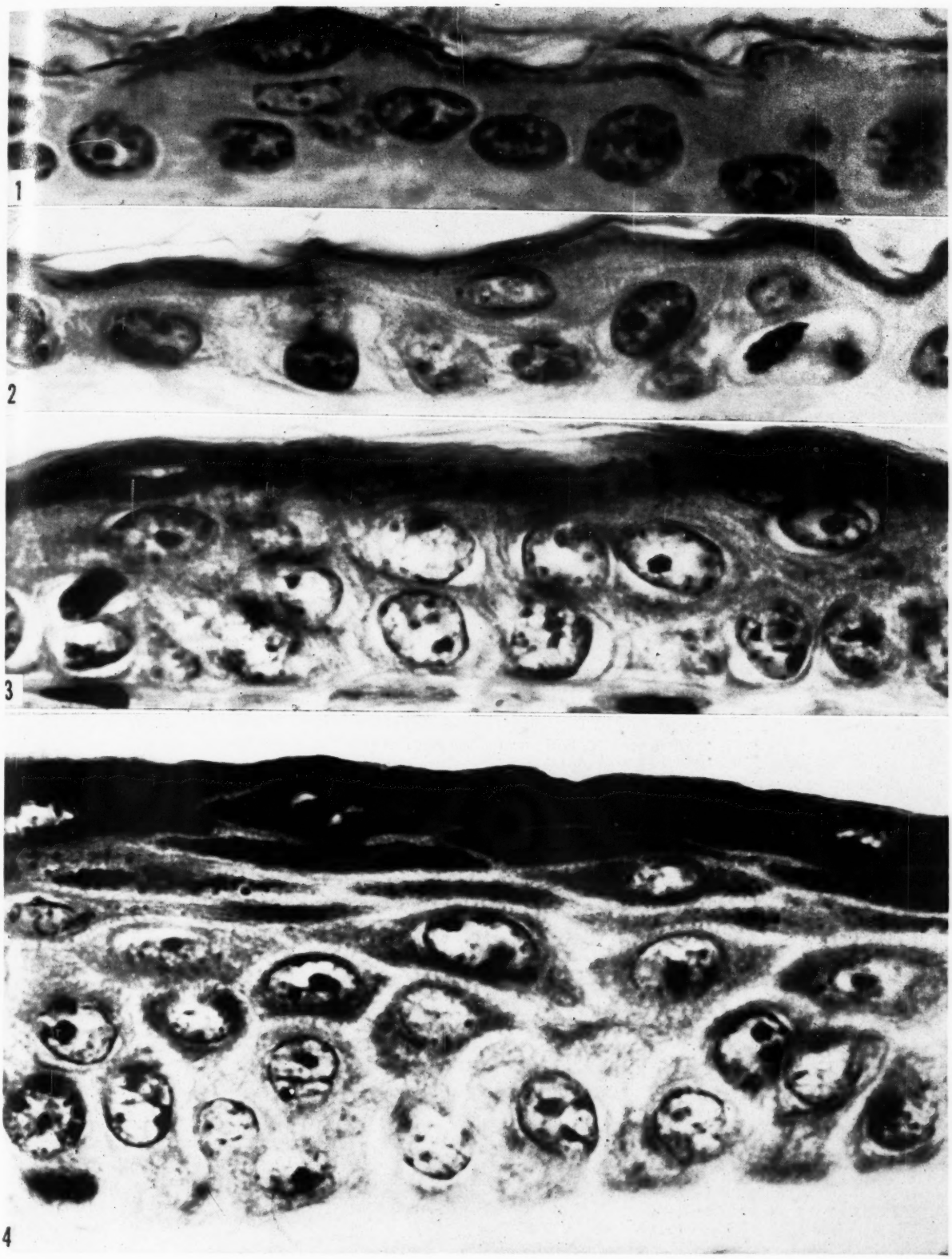
Cells resulting from the application of benzene were arranged in three to four layers during the first few days of the experiment (Fig. 3), and, by the end of the observation period, there were one layer of basal cells, two layers of spinous cells, and one layer of granular cells. Benzene painting apparently did not alter the thickness of the keratinized layers, as compared to the shaved controls. Distinct spines were seen on the basal and spinous cells only up to the 9th day (Fig. 3). They were not observed on the granular cells until the 9th day, after which they disappeared. The cytoplasm of the spinous cells appeared foamy on the 5th and 9th days only. The cytoplasm of the basal cells was more heavily stained than normal after the 5th day, and on the 57th day the cytoplasm of the spinous, and especially that of the granular cells, stained more heavily than normal at any time. The intensity of the stain of the nuclei of the basal cells increased slightly from the 29th to 57th day. This intensity of nuclear staining occurred earlier with the spinous cells and persisted throughout the experiment. It was evident in the granular cells from the beginning through the 29th day.

While there was little change in the proportion of basal cells in division, the proportion in degeneration again rose, from 10 to 22 per cent on the 5th day, but sank thereafter to a low of 4 per cent on the 15th day.

In the spinous cells the proportion in degeneration rose from 1.5 to 12 per cent on the 5th day, but fell to the original level by the 15th day.

Shaved epidermis treated with methylcholanthrene in benzene.—The effect of methylcholanthrene painting on the proportions of epidermal cells was investigated in groups of three or four mice sacrificed after a series of 1, 3, 6, 12, and 24 paintings on 5, 9, 15, 29, and 57 days after the first treatment, respectively.

The total number of cells, cell layers, and layers of keratinized debris of the epidermis increased gradually 5–6 times over the normal during the application of the carcinogen (Fig. 4). Spines were seen between the basal cells at all times, but they reached a maximum on the 9th day. All the spinous cells showed spines which were especially prominent on the 57th day. On the whole, basal cells tend to resemble spinous cells. Spines were also seen between the granular cells on the 15th day and on the 57th day. The cytoplasm of different epidermal cells, except of the granular cells


1
2
3
4

FIGS. 1–4 are photomicrographs at a magnification of 1,900 diameters of longitudinal sections of shaved skin fixed in the Bouin's solution, and stained with hematoxylin and eosin.

FIG. 1.—Normal, unshaved epidermis.

FIG. 2.—Normal (shaved and untreated) mouse epidermis, 8 days after shaving. Note the irregularity in keratohyaline granules and cell size in the granular and spinous layers; the large basal and spinous nuclei, hyperchromatic; and a mitotic figure on the right side.

FIG. 3.—Shaved mouse skin painted with benzene (three paintings, ninth day). Observe the increased thickness, the large nuclei with large nucleoli, hyperchromatism, foamy cytoplasm, distinct intercellular bridges in basal ("temporarily altered basal cells") and suprabasal layers, and large keratohyaline granules.

FIG. 4.—Shaved mouse skin painted with 0.6 per cent methylcholanthrene in benzene (six paintings, fifteenth day). Observe the large nuclei with large nucleoli, distinct intercellular bridges between the basal ("altered basal cells") and suprabasal layers, and very heavily stained, large keratohyaline granules in the granular layer. Note the double nucleoli of the nucleus on the right side and a degenerating basal cell (karyolysis) on the left side.

before the 15th day, was somewhat foamy throughout the experiment. There was a gradual increase in the size of the keratohyalin granules, which became extremely large in the late hyperplasia. The cytoplasm of the basal and spinous cells stained more deeply than normal after the 5th day, while that of the granular cells stained more heavily than normal at all times. The staining of the basal cell nuclei gradually increased in intensity, while that of the spinous and granular cells increased in early hyperplasia but decreased on the 29th and 57th days.

The proportion of cells in division in the basal layer rose progressively from 3 to 27 per cent in 57 days. Throughout this interval the proportion in degeneration rose from 10 per cent to an average of about 15 per cent.

Spinous cells were observed in division for the first time, reaching a peak proportion of 12 per cent by 57 days. The proportion in degeneration rose rapidly from 1.5 to a level of about 15 per cent.

In Chart 1 it will be noted that under methylcholanthrene treatment both basal and spinous cells are grouped as having been altered to a common type (called "altered spinous"), but that the positional distinction between the two has been preserved in the calculations. The fact that the number of basal cells per unit area of basement membrane remains fairly constant throughout all the series is an indication of uniform histological technic.

DISCUSSION

Shaving of mouse skin in itself had no permanent effect; there was only a transient rise in the proportions of spinous and granular cells during the first week or 10 days. Painting with benzene after shaving not only accentuated the transient response but also led to a temporary change in microscopic appearance. Painting with methylcholanthrene resulted in a prompt and continued rise of the spinous cell population, with concomitant rise of the granular cells. Dividing and degenerating cells also became quite numerous. The epidermal hyperplasia following the application of methylcholanthrene was induced in a manner different from that of the usual type of regenerative hyperplasia (such as after shaving), since the increased mitotic activity appears to be permanent and results in structural alterations of all cell types throughout the process of carcinogenesis.

With reference to our introductory remarks, it becomes clear, therefore, that a direct comparison between normal and precancerous epidermis for any chemical constituent is not altogether unwarranted, although neither the proportion of cell types nor their activities remain constant during the carcinogenic process. The comparison must be made in the light of these and other data concerning changes in the epidermal cellular population. Yet it is difficult to conceive of the chemical changes reported being entirely due to shifts in the proportions of cell types, because some properties increase, others decrease, while still others remain constant during epidermal carcinogenesis in mice.

SUMMARY

1. The purpose of this investigation was to study the numerical proportions of different cells in mouse epidermis during methylcholanthrene carcinogenesis, for the purpose of obtaining some information on structural alterations that may condition the reported chemical changes.

2. Groups of Swiss albino mice were shaved and painted with methylcholanthrene in benzene with suitable controls and sacrificed at intervals up to 60 days. Counts were made of the proportions of basal, spinous, granular, dividing, and degenerating cells in the epidermis.

3. Methylcholanthrene induced a high degree of hyperplasia, an increased mitotic rate, and distinct cytological alterations. Basal and spinous cells were transformed to an altered type of spinous cells; granular cells also underwent a change to an altered type.

ACKNOWLEDGMENTS

I am deeply indebted to Dr. E. V. Cowdry and to his staff for their suggestion of the problem and unfailing assistance in its execution.

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Effect of Irradiation on the Universal Reaction in Cancer*

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The universal serologic reaction (1, 2) is believed to be an immunologic indicator of tissue break-down. If this is true, the reaction should show changes resulting from the tissue injury incident to irradiation. That irradiation of animals is followed by changes in their universal reactions is indicated by experiments in progress in this laboratory. The determination whether patients undergoing irradiation show changes was suggested by Dr. Shields Warren of the Atomic Energy Commission. Preliminary studies of universal reactions before and after irradiation of cancer patients will be considered in this article.

Only such data are herein presented that are necessary for a brief introductory report. To determine fully the effect of irradiation on the universal reaction in cancer will require long-range observations of cancer patients undergoing varying degrees of irradiation, with universal reactions repeated at given intervals. However, the fact that the results appear to be of some clinical value suggested the desirability of presenting a report at this time.

The universal reaction.—The universal reaction is a serologic precipitation reaction to lipids based on different NaCl concentrations and different periods of incubation. The universal technic herein considered consists of seven quantitative set-ups. The technical steps of each set-up are similar to those employed in the performance of a quantitative Kahn test, with the exception of the use of different NaCl concentrations and incubation periods. The serum to be examined is first heated for 30 minutes at 56° C. The ratios of serum to diluent employed in each quantitative set-up are: 1:1, 1:2, 1:4, 1:8, 1:16, 1:64, and 1:256. In the first set-up, serial dilutions with serum are made with distilled water; in the second the serial dilutions of serum are made with 0.15 per cent NaCl solution; in the third with 0.6 per cent NaCl solution; in the fourth with 0.9 per cent; and in the fifth, sixth, and seventh set-ups the serial dilutions of serum are made with 1.2, 1.8, and 2.1 per cent NaCl solutions, respectively. Kahn antigen suspension is prepared in the usual manner and permitted to stand for 10 minutes before use. Each of the serial serum dilutions is then mixed with the suspension in a ratio of 6:1, employing 0.15-cc. amounts of the dilutions and 0.025-cc. amounts of the suspension. The mixtures of serum dilutions and suspensions are agitated for 3 minutes in a Kahn shaking machine and the precipitation results read immediately. The results are then further read after 4 and 24 hours' incubation at 5° C.

Table 1 presents an example of a single reading of the precipitation results of the seven quantitative set-ups. Chart 1 illustrates these readings graphically in the form of individual columns on the left side and in the form of a curve arrived at by interpolation on the right side. The cross-hatched area enclosed by the curve and the coordinates represents the zone of precipitation. In the charts in this and in the following article the precipitation results are presented in triplicate curves, comprising all three readings. The graphic presentation of individual columns in Chart 1 is given merely to facilitate the understanding of the relationship between the tabulated precipitation readings and the curves. For economy of space, the curves of Charts 2 and 3 of the universal reactions are presented in miniature form.

EXPERIMENTAL

The general plan of this study was to obtain blood specimens for universal reactions from cancer patients when they reported for irradiation to the Department of Roentgenology, University Hospital, and to submit these to the Serology Laboratory; then to obtain other blood specimens for universal reactions from the same patient about 2 or 3 months after they had received the irradiation therapy, depending on the time when they returned to the hospital for a check-up. In the data to be considered below, only two universal reactions per patient are presented, one

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obtained before and in some instances during irradiation, and the other after irradiation. In later reports it is planned to present data that are being collected on the relationship between irradiation and universal reactions, based on follow-up studies extending for some years.

The blood specimens obtained before and after irradiation were submitted to the laboratory as "unknowns," and, at the time of the performance of the universal technic, the laboratory staff had no knowledge of the clinical status of the patients. As indicated, the clinical data and universal results

TABLE 1

ILLUSTRATIVE PRECIPITATION RESULTS GIVEN BY UNIVERSAL TECHNIC
(Single Reading)

Serum dilutions	Per cent NaCl used in dilutions						
	0	0.15	0.6	0.9	1.2	1.8	2.1
1:256	4						
1:64	4						
1:16	4	2					2
1:8	4	4					4
1:4	4	4	±		±	3	4
1:2	4	4	3		±	4	4
Undiluted	4	4	4		4	4	4

were obtained in different units of the University Hospital.

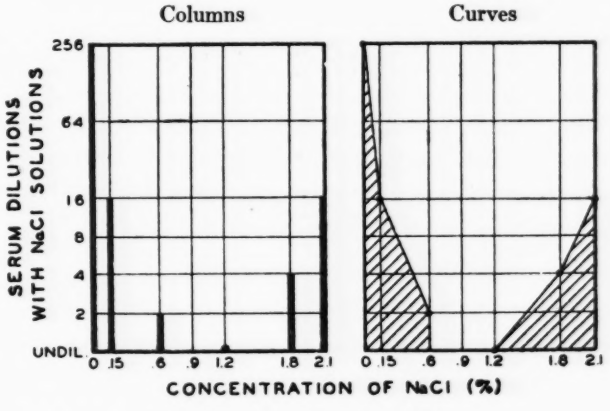
On classifying the universal reactions, it was observed that, following irradiation, certain cancer patients showed increased precipitation compared to the pre-irradiation reactions; others showed no increase, and a few showed a decrease. The question then arose whether a change or lack of change in the universal reactions had any clinical significance. The universal reactions of five cancer patients which showed increased precipitation are presented in Chart 2, together with the clinical histories. The reactions of five other cancer patients which showed no increase in precipitation are presented in Chart 3, with the clinical histories.

It should be pointed out that the universal reaction, based on the technic herein employed, is apparently incapable of showing serologic patterns of increased precipitation over the normal level in nonirradiated cancer patients. Studies of the universal reaction in cancer patients have been carried out in collaboration with the Department of Surgery of the University Hospital during the past 2 years, and, in the serologic examination of more than 300 cancer patients, no definite increase in precipitation over the normal level has been observed; but a tendency toward decreased precipitation has been noted in patients with metastasis. Hence, the increase in precipitation in the universal reaction following irradiation, shown in Chart 2, is interpreted to be the result of the irradiation.

Patient C.J. (Chart 2), with carcinoma of the cervix, was first examined for a universal reaction on August 10, 1950. The patient was given deep x-ray therapy and radium, and, on October 20, 1950, the universal reaction showed increased precipitation. On that date the patient manifested clinically a normal post-irradiation course. Actually, the patient continued to show improvement after that date, as is evident from the clinical history. But no attempt is made here to correlate the continued improvement with the increased precipitation in the universal reaction. The concern here is with the patient's condition on or about October 20, 1950, when the blood specimen for the post-irradiation universal reaction was taken.

Patient L.S., with carcinoma of the endometrium, was first examined for a universal reaction on August 31, 1950. The patient was given deep x-ray therapy and radium and, in addition, underwent a hysterectomy. In this patient the universal reaction showed increased precipitation on December 13, 1950, while the favorable clinical report was made more than 5 weeks later, on January 23, 1951. It is evident from the clinical history, however, that, on the date of the hysterectomy, no recognizable neoplasm could be found on microscopic examination. The improved condition of the patient was thus evident in November, and the increased precipitation in the universal reaction in December.

CHART 1.—Presentation of results in Table 1 in the form of:



In patient H.H., with lymphoblastoma, Hodgkin's disease type, the date of increased precipitation in the universal reaction corresponded to the favorable clinical findings of regression in the cervical mass. This patient developed symptoms of abdominal disease 6 months later, and at that time would undoubtedly have shown another type of universal reaction. In the remaining two patients listed in Chart 2, the increase in precipitation in

UNIVERSAL REACTIONS IN IRRADIATED CANCER

PRECIPITATION INCREASED

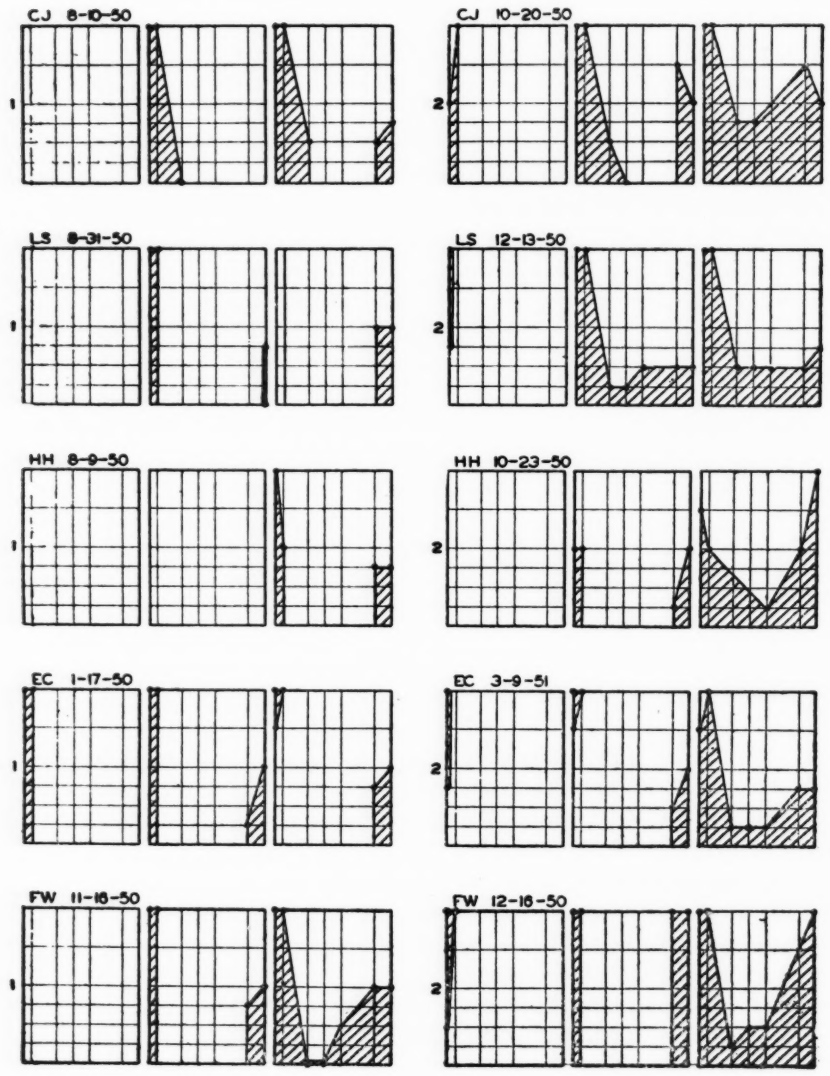


CHART 2

CLINICAL HISTORIES

C.J. FEMALE AGE 59

Chief complaint: Vaginal bleeding, otherwise asymptomatic
Diagnosis: Carcinoma of the cervix; stage III (League of Nations)
Treatment: Deep x-ray therapy: 2,200 r to each of 4 fields 8–9–50 to 9–2–50
Radium: 6,000 mg. hrs. 9–5–50
Univ. reactions: 8–10–50 and 10–20–50
Progress: Radium reaction (necrotic membrane) on cervix 10–20–50
Pelvis otherwise unchanged
Vaginal apex almost healed (pelvis unchanged) 1–29–51
Vaginal apex healed (pelvis unchanged) 4–30–51
Normal post-irradiation course 10–20–50

L.S. FEMALE AGE 67

Chief complaint: Vaginal bleeding
Diagnosis: Carcinoma of the endometrium
Treatment: Deep x-ray therapy: 2,225 r to each of 4 fields 8–29–50 to 9–22–50
Radium: 3,002.33 mg. hrs. 9–25–50
Surgery: hysterectomy 11–18–50; microscopic examination showed no recognizable neoplasm
Univ. reactions: 8–31–50 and 12–13–50
Progress: Normal post-irradiation course 1–23–51. No clinical evidence of neoplasm

H.H. MALE AGE 66

Chief complaint: Mass in neck
Diagnosis: Lymphoblastoma, Hodgkin's Disease type
Treatment: Deep x-ray therapy: 2,050 r to one cervical field directed over a large mass in the left side of the neck
Univ. reactions: 8–9–50 and 10–23–50
Progress: Regression of cervical mass, asymptomatic 10–23–50
Symptoms of abdominal disease, therapy reinstituted 4–23–51

E.C. FEMALE AGE 39

Chief complaint: Vaginal bleeding
Diagnosis: Carcinoma of the cervix
Treatment: Deep x-ray therapy: 2,950 r to each of 2 fields 2,375 r to each of 2 fields 1–15–51, 2–19–51
Radium: 4,200 mg. hrs. 2–24–51
Univ. reactions: 1–17–51 and 3–9–51
Progress: Normal post-irradiation course 3–6–51

F.W. FEMALE AGE 50

Diagnosis: Carcinoma of the cervix; stage II (League of Nations)
Treatment: Deep x-ray therapy: 2,000 r to each of 4 fields 11–13–50 to 12–6–50
Radium: 6,061 mg. hrs. 12–12–50
Univ. reactions: 11–16–50 and 12–16–50
Progress: Normal post-irradiation course 1–29–51

the universal reaction also corresponded clinically to normal post-irradiation courses.

A different relationship between the universal reaction and the clinical post-irradiation course of cancer patients is noted in Chart 3. Patient J.M., with carcinoma of the ovary and with a number of complications, ran a down-hill course clinically in spite of the irradiation. The patient's universal reaction of October 4, 1950, showed reduced precipitation compared to the pre-irradiation reaction of September 1, 1950.

Patient A.S. also showed reduced precipitation in the universal reaction. Although follow-up studies were not possible in this case, the fact that the carcinoma of the larynx was far advanced gives strong indication of the poor clinical condition of the patient.

In the next three cancer patients listed in Chart 3, the universal reactions showed no changes in precipitation following irradiation. In patients J.M. and B.McN., the clinical histories do not indicate a normal post-irradiation course. Patient J.M. was in an especially poor state, with a neoplasm of the left kidney after the right one had been removed. Patient B.McN. had recurrence of carcinoma of the epiglottis with cervical node metastasis. It might have been expected that these two patients, like the two above, would show reduced precipitation in their universal reactions, in view of their poor clinical conditions. The answer might lie in the fact that the degree of precipitation in their pre-irradiation universal reactions was so reduced already as to have very nearly reached bottom, so to speak.

Patient G.S. is of interest. The pre-irradiation reaction of this patient was obtained on August 8th and the post-irradiation reaction on August 31st. This is the shortest period between the pre- and the post-irradiation reactions reported in the present study. The irradiation was completed on August 31st, and the blood specimen for a universal reaction was obtained the same day, which may explain why no increase in precipitation was noted. This is the main reason the universal reaction of G.S. was included in this study—to indicate that a sufficient period following irradiation may be necessary before an increase in precipitation is manifested.

It is evident from the serologic studies of the small group of irradiated cancer patients herewith presented that universal reactions of increased precipitation correspond clinically to normal post-irradiation courses, while universal reactions of decreased precipitation, or of no change in precipitation, correspond clinically to poor post-irradiation courses.

Considerations of the serologic results.—Before considering the results, it will be well to touch upon several aspects of the universal reaction reported elsewhere (1, 2). All human beings and animals tested thus far have been found to show some precipitation in their universal reactions. These reactions are differentiable from one another by differences in precipitation patterns. Different normal individuals generally give precipitation patterns which, to a greater or lesser degree, differ quantitatively from one another, while a given normal individual commonly gives a constant precipitation pattern.

The behavior of the universal reaction in several diseases studied was found to be similar to that of a specific immunity reaction. Thus, in syphilis, malaria, and in early tuberculosis the universal reaction was found to show increased precipitation over the normal level and to revert to that level on recovery. In a given disease, precipitation is marked when the disease is in a moderately active state and is generally at a low level when the disease is in a very high state of activity. For example, while precipitation is often marked in early tuberculosis, it is at a low level in the far advanced and miliary forms of the disease. Similarly, precipitation is marked in moderately advanced lepromatous leprosy, but not in the far advanced form.

Increased precipitation in the universal reaction over the normal level has also been noted following the injection of various substances in rabbits (3, 4). The substances employed were both antigenic (horse serum and killed tubercle bacilli) and nonantigenic (tissue lipids and paraffin oil). The animal irradiation studies in progress have already been mentioned. These studies indicate that irradiation also causes an increase in precipitation in the universal reaction.

As a working hypothesis it is believed that the biologic mechanism of lipid antigen-antibody reactivity, manifested by the universal reaction, is essentially the same in health, in disease, upon injection of various substances, and following irradiation. A common factor associated with this mechanism apparently is tissue break-down, ranging from normal catabolism to marked tissue break-down in disease. Tissue break-down causes liberation of lipids from body cells. Some of these lipids undergo chemical changes which render them foreign to the body and antigenic. Auto-antibodies, formed to these antigenic lipids, are then detected by the universal reaction.

Based upon these considerations, the results obtained in the present study are understandable. It might have been assumed that, since a given

UNIVERSAL REACTIONS IN IRRADIATED CANCER

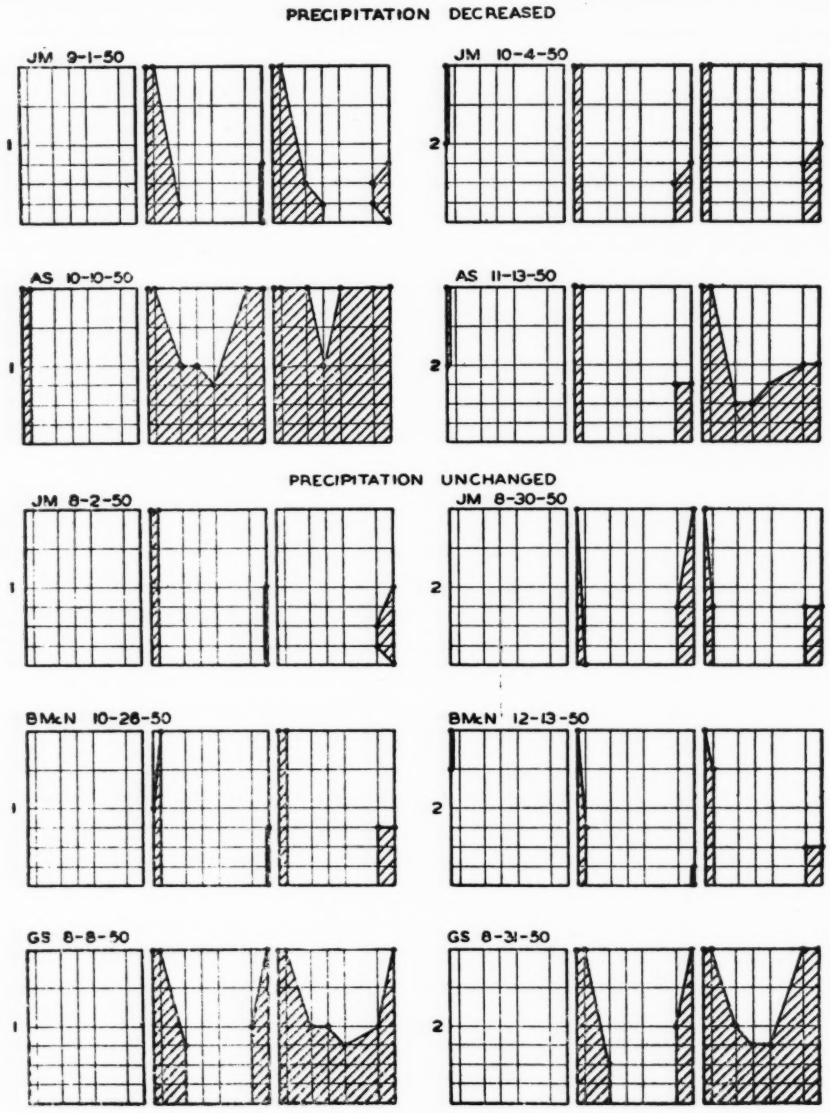


CHART 3

CLINICAL HISTORIES

J.M. FEMALE AGE 46
Diagnosis: Carcinoma of the ovary. Previous surgery and x-ray treatment; colostomy, small bowel obstruction on admission.
Patient in poor condition
Treatment: Deep x-ray therapy; 800 r to each of 8 abdominal fields 8–11–50 to 9–2–50
Univ. reactions: 9–1–50 and 10–4–50
Progress: Down-hill course (considered terminal) 10–4–50
Autopsy 12–13–50; abdominal carcinomatosis

A.S. MALE AGE 45
Diagnosis: Carcinoma of the larynx which was far advanced
Treatment: Deep x-ray therapy: 3,425 r to right cervical field 3,400 r to left cervical field 10–9–50 and 11–13–50
Univ. reactions: 10–10–50 and 11–13–50
Progress: No additional follow-up

J.M. MALE AGE 70
Diagnosis: Renal neoplasm, left (right renal carcinoma removed 1944)
Chief complaint: Hematuria
Treatment: Deep x-ray therapy: 3,000 r to each of 3 fields 8–2–50 to 9–11–50
Univ. reactions: 8–2–50 and 8–30–50
Progress: General condition of patient poor; no observation after 9–11–50

B.McN. MALE AGE 60
Diagnosis: Recurrence of carcinoma of the epiglottis following amputation of the epiglottis 4–7–50, with cervical node metastasis
Treatment: Deep x-ray therapy: 3,775 r to left cervical field 10–25–50 to 12–12–50; 3,825 r to right cervical field
Univ. reactions: 10–26–50 and 12–13–50
Progress: No change as of 12–13–50; no evidence of neoplasm 5–11–51

G.S. FEMALE AGE 29
Chief complaint: Vaginal bleeding
Diagnosis: Carcinoma of cervix; stage I (League of Nations)
Treatment: Deep x-ray therapy: 2,000 r to each of 4 pelvic fields 8–8–50 to 8–30–50
Radium: 6,000 mg. hrs. 8–31–50
Univ reactions: 8–8–50 and 8–31–50
Progress: No clinical evaluation possible as of 8–31–50

degree of irradiation is likely to produce the same degree of tissue injury in different patients, the lipids liberated and the antibodies produced in these patients would be such as to lead to universal reactions of similar intensity. Actually, because the behavior of the reaction is that of an immunity reaction, those irradiated patients who are going down-hill clinically could not be expected to show the same capacity for antibody production as those who are improving.

The increase in precipitation in the universal reaction, manifested by the irradiated cancer patients listed in Chart 2, indicates that the injury to the cancer tissue caused by the irradiation resulted in improvement of the patients sufficient to enable them to respond with increased antibody production to liberated lipids. The lack of increase in precipitation in the universal reaction, manifested by the irradiated cancer patients listed in Chart 3, indicates that the irradiation apparently failed to improve the patients sufficiently to enable them to respond with increased antibody production to the liberated lipids. A reasonable explanation for this inability to increase their antibody production is the general debility of the patients, due in most instances to metastasis. Needless to say, lack of increase in precipitation will also be noted when insufficient time is allowed for increased antibody production following irradiation, as in patient G.S. (Chart 3).

Superimposed disease or immunizing injections might affect the degree of precipitation in the universal reaction in irradiated cancer patients. It is conceivable that in a certain cancer patient the irradiation might be clinically successful, but, instead of increased precipitation in the universal reaction, no increase might be noted because of the patient's inability to increase the production of antibodies above the pre-irradiation level as a result of severe illness unrelated to cancer. However, an increase in precipitation in the universal reaction of a cancer patient following irradiation, even in the presence of superimposed disease or immunizing injections, might suggest a capacity to increase the production of antibodies. This capacity in turn would indicate improvement, since it apparently would be absent in metastasis.

It is evident that only by means of extensive and prolonged studies of the universal reaction in irradiated cancer patients, with repetition of the reaction every few months, will it be possible to determine the extent to which it may serve as an aid to the clinician in interpreting the results of irradiation therapy in cancer.

SUMMARY

Preliminary studies of universal reactions before and after irradiation of cancer patients led to the following results:

1. Patients whose universal reactions exhibited a rise in precipitation following irradiation were found to manifest clinically a normal post-irradiation course.

2. Patients whose universal reactions exhibited no rise or a decline in precipitation following irradiation were found to be in a very poor state clinically and did not manifest a normal post-irradiation course.

ACKNOWLEDGMENT

The authors wish to thank Miss Ellen Blue for technical assistance.

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Effect of Irradiation on the Universal Reaction in Polycythemia Vera*

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The preliminary studies herewith presented are part of a project to investigate the behavior of the universal reaction in various blood diseases, in collaboration with staff members of the Simpson Memorial Institute. In this article data will be presented on changes in the universal reaction of patients with polycythemia following irradiation, in relation to clinical changes.

EXPERIMENTAL

Blood specimens were obtained from patients with polycythemia, at the time they reported to the Simpson Memorial Institute for treatment with radioactive phosphorus (P^{32}), and were submitted to the Serology Laboratory for universal reactions. The patients then returned for a check-up, generally from 1 to 4 months after they had received the irradiation therapy, and blood specimens were again obtained for universal reactions. As in the previous study of the effect of irradiation on the universal reaction in cancer (1), the specimens were submitted to the laboratory as "unknowns." After the specimens were examined with the universal technic, the results were correlated with the clinical findings.

Chart 1 presents universal reactions of six patients with polycythemia in the course of treatment with radioactive phosphorus. Two universal reactions are given of each patient, and the second, in each instance, shows an increase in precipitation over the first. In each instance, also, the patients show clinical improvement at the time of the second reaction. Thus, KA, the first patient listed, gave a universal reaction of very little precipitation on October 10, 1950, when the polycythemia was not under control and she needed therapy; she also needed therapy a month later, on November 6th. But on January 3, 1951, the disease was controlled, and at that date the universal reaction showed an increase in precipitation over the reaction of October 10, 1950. The same direct relationship between the control of the polycythemia and the increase in precipitation in the universal reaction applies to the other five patients.

Chart 2 presents universal reactions of six other patients with polycythemia in the course of treatment with radioactive phosphorus, in which no increase in precipitation is noted on the second examination. Indeed, some reactions show a small decrease in precipitation. It is evident from the clinical histories that in none of the patients is the disease under control. Thus, NK, the first patient listed, needed therapy not only when the first universal reaction was obtained but also when the second reaction was obtained. In the second patient listed, BS, the polycythemia was under control at the time when the first universal reaction was obtained, but the erythroid values had risen at the time when the second reaction was obtained.

Briefly, increased precipitation in the universal reaction corresponds to clinical improvement of the irradiated polycythemia patients, while no increase in precipitation corresponds to no clinical improvement. A similar relationship between the universal reaction and the clinical status of irradiated cancer patients was reported in the preceding article (1). It would thus appear that the irradiation of either cancer or polycythemia patients exerts similar effects on the universal reaction.

The increase in precipitation in the universal reaction of the polycythemia patients in whom the disease is controlled by the irradiation is explained on the assumption that the tissue injury incident to the irradiation liberates antigenic lipids which call forth an increase in the antibody response beyond the normal level. But the lack of increase in precipitation in the universal reaction of the patients in whom the disease is not controlled and who require further irradiation therapy is difficult to explain. It cannot be said that these polycythemia patients are unable to produce antibodies to

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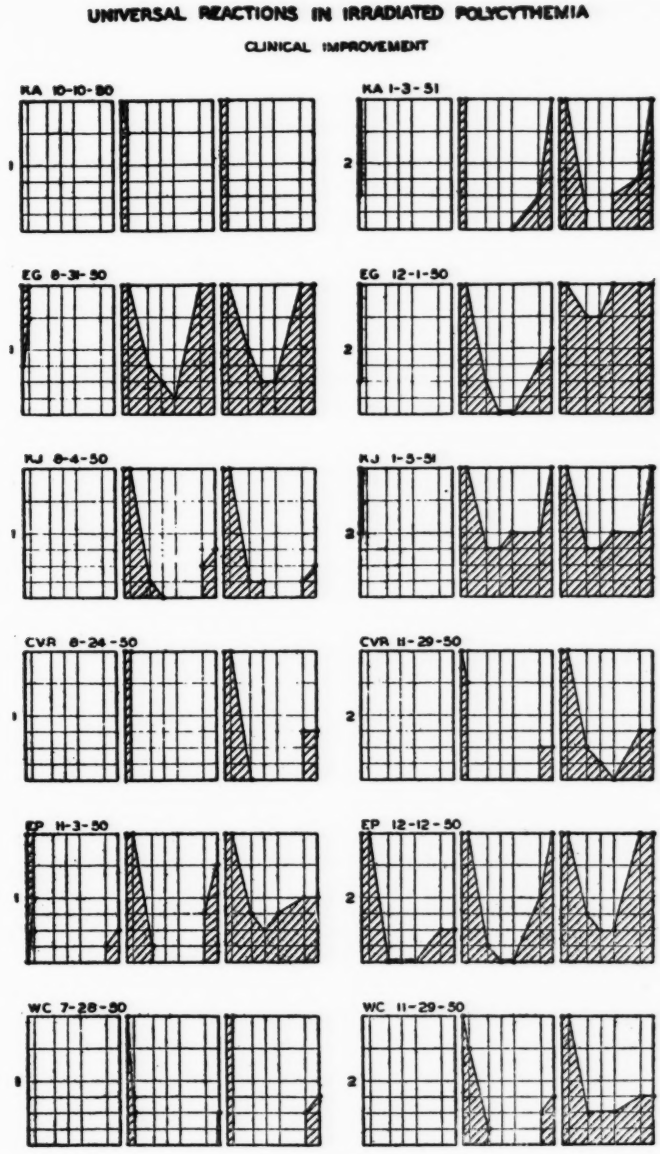

CHART 1

CLINICAL HISTORIES

K.A. FEMALE AGE 43
Diagnosis: Polycythemia vera
Treatment: Has been followed since 11–3–48. Received a total of 11.8 mc. of P^{32} in 3 treatments. On 10–10–50 and 11–6–50 needed further treatment
Univ. reactions: 10–10–50 and 1–3–51
Progress: 1–3–51 disease controlled

E.G. FEMALE AGE 51
Diagnosis: Polycythemia vera and essential hypertension
Treatment: Has been treated since 3–13–48. Received a total of 21.7 mc. of P^{32} in 5 treatments
Univ. reactions: 8–31–50 and 12–1–50
Progress: 8–31–50 disease controlled
12–1–50 disease controlled

K.J. FEMALE AGE 55
Diagnosis: Polycythemia vera
Treatment: 8–4–50 seen first time. Received a total of 7.3 mc. of P^{32}
Univ. reactions: 8–4–50 and 1–5–51
Progress: 1–5–51 disease controlled

C.V.R. FEMALE AGE 66
Diagnosis: Polycythemia vera; hypertensive cardio-vascular disease; right hemiparesis; post-thrombotic
Treatment: Has been followed since 10–9–48. Received a total of 12.8 mc. of P^{32} in 3 treatments. On 8–31–50 treated with 5.6 mc. of P^{32}
Univ. reactions: 8–24–50 and 11–29–50
Progress: 11–29–50 disease controlled

E.P. FEMALE AGE 65
Diagnosis: Polycythemia vera and diabetes mellitus
Treatment: Has been followed since 3–21–49. Total body irradiation 4–21–50 and 10–18–50
Univ. reactions: 11–3–50 and 12–12–50
Progress: 11–3–50 disease controlled
12–12–50 disease controlled

W.C. MALE AGE 72
Diagnosis: Polycythemia vera
Treatment: Has been followed since 7–5–50. No treatment. On 7–28–50 treated for the first time with 5 mc. of P^{32}
Univ. reactions: 7–28–50 and 11–29–50
Progress: 11–29–50 improving

UNIVERSAL REACTIONS IN IRRADIATED POLYCYTHEMIA

NO CLINICAL IMPROVEMENT

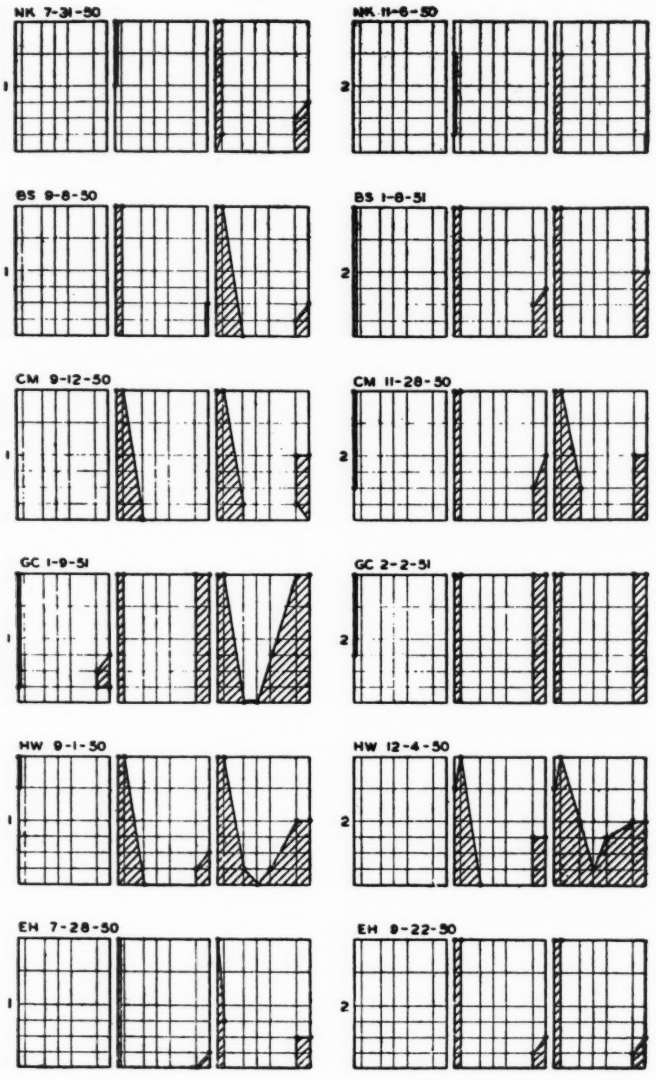

CHART 2

CLINICAL HISTORIES

N.K. FEMALE AGE 51
Diagnosis: Polycythemia vera
Treatment: Has been followed since 11–9–45. Treatment with total body irradiation 4 times from 1945 to 1947. Received total of 25.7 mc. of P^{32} in 5 treatments between 1947 and 1950
Univ. reactions: 7–31–50 and 11–6–50
Progress: 7–31–50 needed therapy
11–6–50 needed therapy

B.S. MALE AGE 49
Diagnosis: Polycythemia vera
Treatment: Has been followed since 3–8–49. Received a total of 5.9 mc. of P^{32}
Univ. reactions: 9–8–50 and 1–8–51
Progress: 8–9–50 disease controlled
1–8–51 erythroid values had risen

C.M. MALE AGE 58
Diagnosis: Polycythemia vera
Treatment: Has been followed since 12–8–49. Received a total of 19.2 mc. of P^{32} in 3 treatments
Univ. reactions: 9–12–50 and 11–28–50
Progress: 9–12–50 disease controlled
11–28–50 erythroid values had risen

G.C. MALE AGE 54
Diagnosis: Polycythemia vera; old myocardial infarction
Treatment: Has been followed since 10–3–50. No previous treatment
Univ. reactions: 1–9–51 and 2–2–51
Progress: 1–8–51 disease active
2–2–51 disease active

H.W. MALE AGE 61
Diagnosis: Polycythemia vera
Treatment: Has been followed since 6–22–44. Total body irradiation 1944 and 1946. Received total of 24.4 mc. of P^{32} in 5 treatments between 1945 and 1949
Univ. reactions: 9–1–50 and 12–4–50
Progress: 9–1–50 disease controlled
12–4–50 needed therapy

E.H. MALE AGE 38
Diagnosis: Polycythemia vera
Treatment: Has been followed since 1–13–49. Received total of 24.6 mc. of P^{32} in 6 treatments between 1949 and 1950
Univ. reactions: 7–28–50 and 9–22–50
Progress: 7–28–50 disease active; no P^{32} administered because platelets depressed
9–22–50 disease active; no P^{32} administered because platelets depressed

lipids due to debilitation, since they are in a fair state of health even when the disease is not fully controlled. Hence, the tissue injury incident to the irradiation and the resulting increase in antigenic lipids might be expected to call forth a corresponding increase in the antibody response whether or not the patients show improvement.

It is possible that the lack of increase in precipitation in the universal reaction of the irradiated patients, when the polycythemia is not controlled, is associated with red cell production which may in some way interfere with antibody formation.

SUMMARY

Preliminary studies of universal reactions before and after irradiation of patients with polycythemia vera led to the following results:

1. In the patients in whom the universal reactions showed a rise in precipitation following irradiation, the polycythemia was found to be under control.

2. In the patients in whom the universal reactions showed no rise in precipitation following irradiation, the polycythemia was found to be in an active state and not under control.

ACKNOWLEDGMENT

The authors wish to thank Miss Ellen Blue for technical assistance.

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Differential Invasion of Embryonic Chick Tissues by Mouse Sarcomas 180 and 37

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INTRODUCTION

The method of tumor transplantation to the extra-embryonic membranes of the chick embryo was inaugurated by Murphy and Rous (8, 9), who successfully transplanted the Rous chicken sarcoma to the chorioallantoic membrane. Shortly thereafter, Murphy (6, 7) made the first transplantation of mammalian tumor (Jensen rat sarcoma) to the extra-embryonic membranes of the chick. Since that time, the technic has been elaborated, and it is now widely used for the propagation and maintenance of tumors (11).

The compatibility of mouse tumors and chick embryos suggested to Bueker (2) the possibility of the implantation of mouse sarcomas into the embryo proper, in connection with certain neuro-embryological problems. Bueker implanted small pieces of mouse Sarcoma 180 into the flank of 3-day embryos and found that the implanted tumor grew rapidly. It was invaded by sensory nerves of the chick, resulting in hyperplasia of the corresponding ganglia. The experiment was continued in this laboratory by Levi-Montalcini and Hamburger (5), who confirmed and extended Bueker's findings. These authors also implanted mouse carcinomas dbrB and C3HBA and found that neither would grow intra-embryonically, although Coman (3) has successfully cultured mouse carcinomas on the extra-embryonic membranes of the chick.

Levi-Montalcini and Hamburger reported some observations on the growth of the sarcomas and their infiltration of tissues of the host embryo. A distinct affinity of the neoplastic tissue for certain organs, particularly the mesonephros, was evident; other structures, such as the metanephros, skeletal elements, and nerve tissues were not invaded, although they were equally accessible to the tumor. This phenomenon of selective affinity and destruction seemed to deserve a more detailed investigation. In this connection, the study of the growth pattern of the tumor became a point of interest. The present investigation is concerned with the period from tumor implantation, at 2½ days of incubation, to the ninth day, by which time the tumor has become well established at its permanent site.

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MATERIALS AND METHODS

The original stock of mouse Sarcomas 180 and 37 (hereafter referred to as S180 and S37) was obtained from the Jackson Memorial Laboratory at Bar Harbor, Maine; it was carried in closely inbred white mice. It was maintained in our laboratory in a colony of white mice of unknown genetic background.

Much of the material used in the present study was loaned by Drs. Levi-Montalcini and Hamburger; this was supplemented by a number of similar experiments by the author.

The operative technic was described by Hamburger (4) and Levi-Montalcini and Hamburger (5). Tumors which had grown from 4 to 14 days in the mouse and were readily palpable were removed to sterile saline. Pieces of about 1 c. mm. were selected from a healthy, non-necrotic portion of the tumor. The transplant was placed in a small slit in the right body wall, lateral to the somites and at the base of the wing- or hind-limb bud (Chart 1). It was exposed at the surface and extended into the body cavity in most cases, being held in place by the tension of the walls of the wound. Implantation was always made at 2½ days; the age of the cases as recorded in these experiments refers to age in days of incubation of the chick host.

Embryos were fixed at regular intervals from 12 hours to 7 days after operation. They were fixed with Bouin's fixative, stained *in toto* with hematoxylin (B. Wenger's modification of the Heidenhain technic [12]), and sectioned at 10 μ. Some were counterstained lightly with Eosin B.

RESULTS

Some General Characteristics of the Tumor

The two sarcomas used in this study appeared to be histologically identical. During the period under consideration (i.e., through the ninth day of

incubation), they invaded the same organs in essentially the same way. Some differences in *degree* of invasiveness, which may show a trend toward greater activity by S37, will be discussed later. Except where otherwise noted, the following descriptions refer equally to both sarcomas, representative cases being selected without prejudice.

The tumor mass had characteristic properties which made its identification certain in all instances. The tumor cells were larger than the host cells. The tumor nuclei, and to a lesser extent the cytoplasm, stained more deeply in hematoxylin than did most normal chick tissues. A higher percentage of mitotic figures was seen in the tumor tissue than in most tissues of the chick embryo, and many of them were atypical. The tumor mass often included necrotic areas and nests or strands of host cells. Ingrowth of nerves into the tumor, which began at 6½ days, was the subject of a detailed investigation by Levi-Montalcini and Hamburger (5). Blood vessels within the tumor were distinctly recognizable by 5½ days.

It was not always easy to identify individual neoplastic cells which had migrated out from the main tumor mass. The large size of the tumor cells, their previously mentioned differential staining capacity, and large, granular nuclei, which were often polymorphic, were sometimes found to be useful characteristics for their identification.

Growth Pattern of the Tumor

For a few days after implantation in the chick embryo, the tumor tissue underwent considerable necrosis. During and following this necrotic period, surviving tumor cells divided rapidly, infiltrated host tissues, and established centers of growth in certain nearby organs. It is convenient to divide the period under consideration into a *regressive phase* (3–5½ days), characterized by the necrosis, and a later *phase of active growth* (from 5 days on). The two phases are not sharply separated but overlap considerably in 4- to 6-day cases. It should be emphasized that active growth of the tumor did not cease at 9 days, but continued as long as the

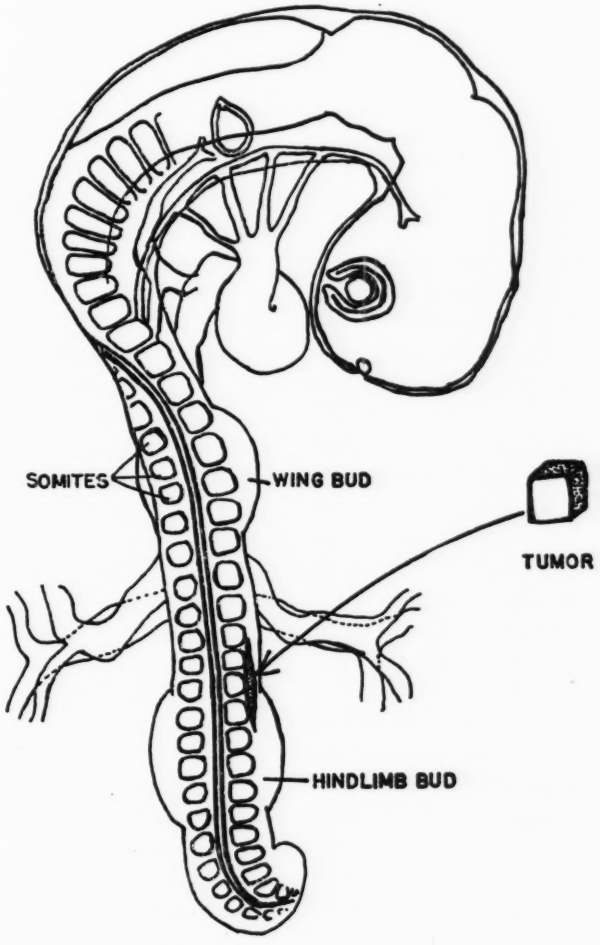


Chart 1.—Diagram of operations (explanation in text)

chick remained alive, i.e., until near the end of the incubation period in some cases.[1] The following description of the growth pattern of the tumor is derived from our study of a closely staged series of cases (Table 1).

Whereas the tumor at first extended outside the body wall, the incision wound usually closed over within the first day after operation. The tumor remained centered in the somatopleure during the regressive phase; its relatively few viable cells migrated out for short distances within the mesenchyme of the body wall.

The necrotic area always lay in the center of the tumor mass, surrounded by a fringe of healthy, dividing cells (Figs. 1, 2). It had two components: (*a*) a hyaline substance in which no cell boundaries could be distinguished; and (*b*) small basophilic granules, usually clustered in the center of the hyaline area. These had the appearance of droplets

TABLE 1

SUMMARY OF CASES

	Age (days of incubation)							
	3–3½	4–4½	5–5½	6–6½	7	8	9	Total
Series S180	3	4	10	7	3	2	0	29
Series S37	5	4	7	3	4	1	3	27
Totals	8	8	17	10	7	3	3	56

of chromatin, possibly the remnants of cell degeneration, or possibly pyknotic nuclei.

The cells at the periphery of the tumor were very irregular in shape; they often showed blunt pseudopodia or pointed cytoplasmic extensions. They were found at increasing distances from the tumor center in 3- to 5-day cases. These characteristics suggested that the marginal cells possessed a high degree of amoeboid motility and reached new sites by active migration.

The tumor mass as a whole had a loose texture in early stages. All its cells were interspersed with host cells. As the necrotic region decreased in size and finally disappeared (at 6 days in almost all cases), more densely arranged tumor cells filled its position in the center of the tumor mass. These cells lost their irregular outlines and became spherical or spindle-shaped, probably due to dense packing.

At 5 days of incubation, the expanding tumor had kept pace with the increase in the width of the body wall and with the considerable distension of the coelomic cavity of the chick (Chart 2). As a rule, the tumor was still centered in the body wall. It was farther from the epidermis than in earlier stages, although in several cases a strand of cells extended to the incision scar. The medial side of the tumor now formed a broad bulge into the coelom (Fig. 1), and occasionally locally filled the right side of the body cavity. Through this extension, contact was established with various visceral organs, especially the developing mesonephros.

In this way, a bridge was formed from the body wall across the coelom (Fig. 2). Many migratory tumor cells reached the mesonephros in this way; here they infiltrated the interstitial spaces so that soon all mesonephric tubules, at the level of the tumor, were surrounded by tumor tissue. Other structures, like the liver and gut wall, were superficially invaded.

From approximately 6 days on, the bulk of the tumor shifted inward to the right mesonephros and coelom, where it became established; it proliferated extensively and progressively invaded adjacent host tissues (Chart 2). One had the impression that once a considerable mass of tumor cells had become lodged in the mesonephros, the tumor became stationary at this site. In most cases in which the tumor had shifted to a more medial position, a narrow strand of cells still extended across the body wall toward the point of implantation.

In older cases the tumor mass was often extremely large and very dense throughout. However, the cells at its margin remained scattered and apparently migratory.

With a single exception, all cases of S180 were smaller and somewhat less invasive than those of S37. It is also interesting to note that necrosis ended sooner in the latter series (see Table 2, column 2). However, it should be noted that in the period covered by this investigation (i.e., through 9 days of incubation) these differences were not great, and showed, at most, only a trend. Our data showed no significant differences in the number and type of structures invaded by the two tumors, the difference indicated being one of *degree* of invasiveness only. Levi-Montalcini and Hamburger (5) reported that in older cases (beyond 9 days) S37 is much more invasive than is S180, especially with respect to musculature and connective tissue. This behavior of S37 does not become apparent until later stages, beyond the period covered by this investigation.

Differential Invasion

A systematic survey was made of all structures within the range of the growing tumor, using a series of arbitrary estimates of the degree of invasion or refractoriness (see legend of Table 2). It will be noted that the degree of invasion of the mesonephros, as well as the total number of structures invaded, increased steadily during the phase of ac-

[1] Levi-Montalcini, personal communication.

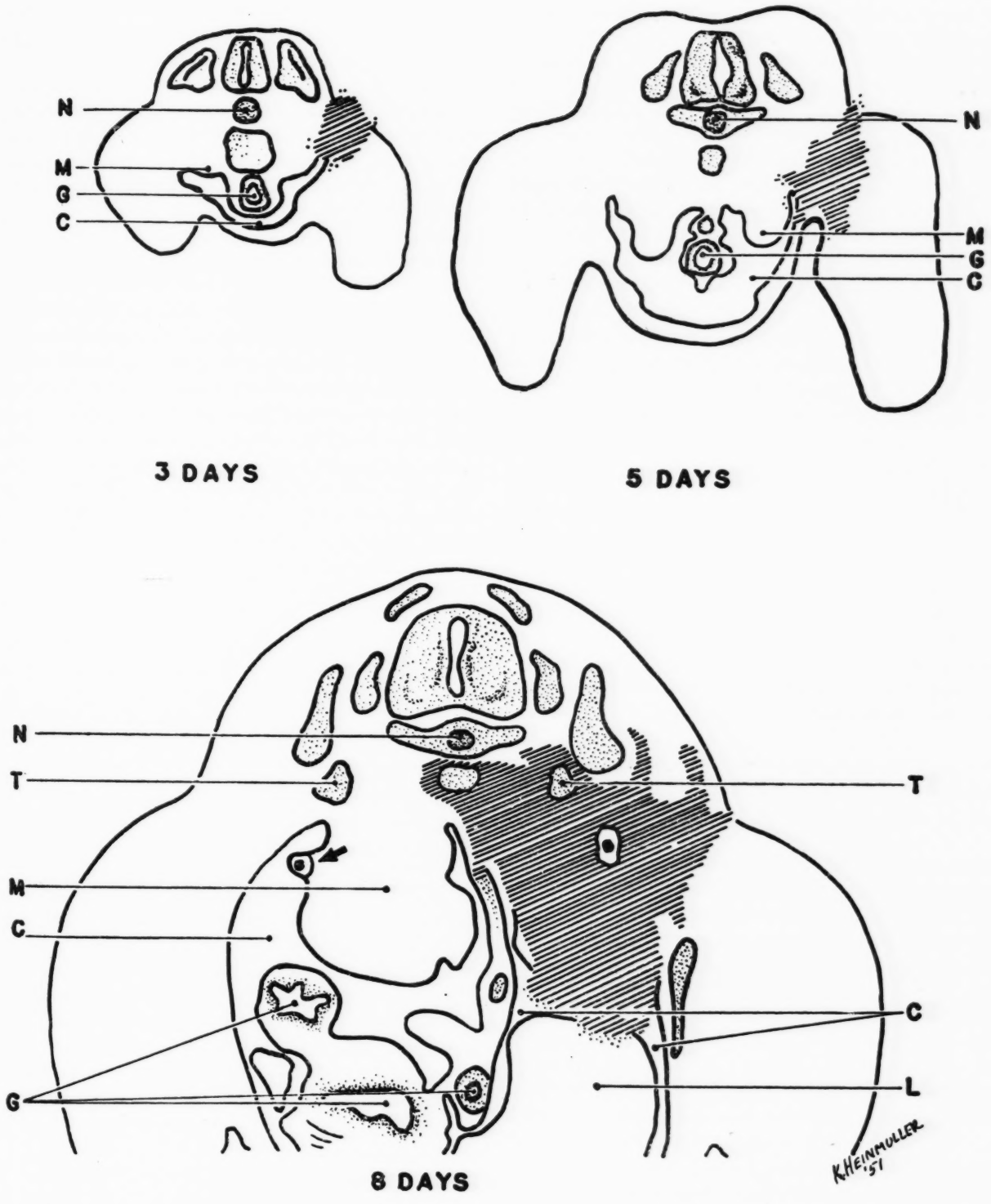


CHART 2.—Various stages in the growth pattern of the tumor in the chick embryo. The tumor area is represented by diagonal hatching. Abbreviations: A, dorsal aorta; C, coelom; G, gut; L, liver; M, mesonephros; N, notochord; S, tumor; T, metanephros; an arrow indicates the position of the Müllerian duct.

TABLE 2

DEGREE OF INVASION OF HOST STRUCTURES BY THE TUMOR

HOST EMBRYOS*	AGE (IN DAYS OF INCUBATION)	NECROSIS	MESONEPHROS: Interstitial spaces	MESONEPHROS: Tubules	MESONEPHROS: Wolffian duct	BODY WALL	GUT MESENTERIES	GONAD	MÜLLERIAN DUCT	YOLK SAC	ADRENAL GLAND	LIVER	SKELETAL ELEMENTS	GUT WALL (SPLANCHNOPLEURE)	AIR SACS	LUNG
37-K1	3½	x	++	00		+				+				+		
37-K6		x	++	00		+								+		
37-K7		x				+								++		
37-K11		x				+								+		
37-K15		x				+										
37-K23	4	x				+								+		
37-K90		x				+++								+		
37-K98		x				+++								+		
37-34		x	++	0	++	++++	++			++				+		
37-35	5	x	++	00	000	++++	++	++		++						
37-41		x	+		++	++++				++						
37-47			++++	+++	++	++	+						++			
37-49†			++++	++++	+++	++	++	++	000	++		+				
37-11	5½		+++	+000	00	++++	++						+++			
37-12			++++	++0	00	+++	+			++						
37-46			+++	++00	000	+++	++	+								
37-51	6½		++++	+++	+++0	+++	+	+	++00			+	00	+		
37-53			++++	++++	+++	++	++	+	00	+	+++	++	++00	++	+	
37-54			++++	+++	0000	+++	+++	+	++00	++			+000	+		+
37-1†	7		++++	+++	++++	+++	++		++++			++	000			
37-2†			+++	00		+++						+	000			
37-3†			++++	++++	++++	+++	++++	++++	0000	+	++	++	000		++	++
37-10†			++++	++++	++++	++	+		0000	+			00			
37-K41	8½		+++	+++			++++				+000		00		++++	
37-7†	9		++++	++++				+++0	++++			+	0000			
37-8†			++++	++++	++++	++++	++	++++	0000	+	+++		0000	+	+	+
37-9			++++	++++	++++	+++	++	+	000	++	+++	+	+000			
180-K8R	3	x				+										
180-K8L		x				+										
180-K10	3½	x				+								+		
180-K2	4	x				+								+		
180-K6		x				+								+		
180-K3		x				+								+		
180-K7		x				+								+		
180-32	5	x	+++	+0	000					++						
180-34†			++++	+++	000		++	++++			++					
180-59†		x	+++	++		++			000		++	+		+		
180-71			+++	+++	++00		+							++		
180-72		x	++	+00	000		++++									
180-33	5½	x	++++	++++	++00	+++	++		++++							
180-36		x	++	+	000	++++			00							
180-57†			+++	++	000	+++			0000				00			
180-58†			+++	++	0000	+++			0000				+00			
180-76†			++++	++++	0000	++	++	++	0000			++				
180-75	6		+	+	00	++			0000		++	++	000			
180-77A†			++++	+++	0000			++	++00					+		
180-77P			+++	++	0000		+++									
180-78L		x	+++	+++	00	+++			++++							
180-113	6½		+				++		++00	++			000			
180-114			++	0	00	++	+++		000	++			+00		++	
180-115			++	++	000	+++							00			
180-5†	7		++++	++++	++++	+++	++	++++	0000		++	++	000			++
180-119			++	+	0000	+++	++		0000				000		++	
180-120			++++	+++	0000			++++	+++				0000	+		
180-124	8		+++	++	0000	+++	++		++00			++	000	+	+	
180-125			++++	++++	++++	++	++	+	++++		+		000			

\+ to ++++: invasion by a few tumor cells *to* extensive invasion and destruction.

0 to 0000: a few tumor cells in the region of, or surrounding the structure *to*, structure completely imbedded in the tumor mass but not invaded or destroyed by it.

Each rating was made at the level of maximum invasion and destruction, or maximum refractoriness, respectively.

* Sarcoma 37 or 180 denoted by chick number.

† Denotes transplants into wing level.

tive growth (after 5 days). Invasion of organs on the left side, which followed after extreme invasion and destruction on the right side, was not recorded on the table.

Of all structures adjacent to the tumor, the mesonephros stood out as the most severely affected organ (Figs. 4–6). It was often completely replaced by tumor along part of its length, and usually became the center at which the main tumor mass established itself in later stages. Other tissues showed more or less resistance to tumor invasion, varying from complete refractoriness to temporary resistance.

Mesonephros.—The mesonephros of the chick embryo begins to differentiate at about 3 days and develops progressively in an antero-posterior direction. When it begins its functional activity at about 5 days, only its cranial portion is differentiated. At approximately the end of the second week of incubation, the metanephros begins its excretory function, and the mesonephros is degenerating (10). Some of our cases showed severe destruction in the right mesonephros as early as at 7 or 8 days (Fig. 4); evidently, the remaining intact kidney was capable of taking on any extra excretory activity required.

When invasion began at about 5 days, tumor cells first migrated into the interstitial spaces, until only the tubules and a few glomeruli remained imbedded in the dense tumor mass (Fig. 5). Subsequently, the tubules disappeared, and in later stages some sections showed no remnants of the right mesonephros (Fig. 6). It is interesting to observe that the Wolffian duct often remained intact even after most of the tubules had been destroyed (Fig. 6). On the other hand, in a few cases the first invading cells entered the mesonephros near the Wolffian duct, and the latter was occluded early. In case S180-33, as a result of complete occlusion of the duct by the tumor, the Wolffian duct was greatly distended anteriorly and exceeded in size the entire left (control) mesonephros. Boyden (1) described similar distension in cases in which he removed the growing tip of the Wolffian duct, thus preventing it from establishing contact with the cloaca.

As is shown in Table 2, the mesonephros seemed to be equally invaded, whether the tumor was implanted in the hind-limb or wing-level. At 6 days, when invasion of that structure was evident, tissue which was to form mesonephros extended from the wing- to hind-limb level and was easily accessible to tumors implanted at either site. However, some structures in the following group (e.g., liver, lung) were more accessible to wing-level implants and were invaded in most cases only by tumors implanted at the more anterior site.

Other structures adjacent to the tumor.—The tumor in its expansion encountered many other structures which it might have invaded. Apparently, none of these were as favorable for tumor growth as was the mesonephros. One group of organs (liver, lung, gut wall, and coelomic walls) became superficially invaded, but the tumor did not establish itself in them. A second group (gonad, adrenal, Müllerian duct, muscle) were temporarily refractory, but they were often eventually invaded and destroyed. A third group of structures (nerves, ganglia, blood vessel walls, cartilage, metanephros) were often adjacent to the tumor or imbedded in its mass, but seemed to be highly refractory; they were almost never invaded by tumor cells in the period up to 10 days. We shall use this tentative grouping in the following description.

First group.—Between 5 and 6 days the *liver* was close to the expanding tumor and exposed to invasion. Of 40 embryos of 5 days or older, 13 showed superficial liver invasion (Fig. 4). Since the liver was more accessible to tumors implanted at the wing level, 10 of the 13 cases were from wing-level implants. It is significant that older cases showed no deeper penetration of the liver by the tumor than did the youngest. Although the tumor probably destroyed and replaced a small amount of liver tissue, histological evidence of this process was not seen.

The edge of the splanchnopleure of the gut wall was similarly invaded superficially in a few cases. Again, there was no progressive increase in the amount of neoplastic tissue at this site.

Altogether, only four cases displayed a slight superficial invasion of the lung. Again, neither expansion nor progressive tumor growth followed the initial invasion. Only few tumor cells were found in the loose mesenchymous tissue between bronchiole buds.

The body wall was placed in this group, because it never became a major center for tumor growth. However, in all cases a number of tumor cells remained in the body wall, which was the site of original implanation, and occasionally tumor cells crept along the somatopleure (Fig. 4).

Second group.—Gonad, adrenal, Wolffian duct, Müllerian duct, and muscle were invaded slowly by the tumor. Marginal tumor cells sometimes migrated between dense cords of gonad or adrenal cortical cells, or around the wall of the Wolffian duct or Müllerian duct (Fig. 5), without at first penetrating these structures. In older embryos with large tumors, the tumor cells sometimes penetrated and destroyed these structures locally.

† Denotes transplants into wing level.

The Müllerian duct retained temporary refractoriness longer than the other structures. In some cases it remained the only intact structure within the dense center of the tumor after the right mesonephros had been entirely destroyed (Fig. 5) locally. However, in eleven advanced cases even the Müllerian duct had been destroyed at the level of the tumor.

The tumor never became well established in any of the structures in either of the above groups. Rapid proliferation evidently did not follow as it did when cells infiltrated the mesonephros.

The secondary centers of tumor growth to which we refer above did not conform strictly to the commonly adopted definition of metastases; they did not result from the transport of cells to distant sites, but were always contiguous with the primary tumor. In older cases of this series (beyond 9 days), true metastases have been observed by Levi-Montalcini.[2]

Third group.—These structures were characterized by complete refractoriness to the tumor. It has already been noted that nerves, ganglia, cartilage, blood vessels, and metanephros often became surrounded by, or imbedded in, the expanding tumor mass (Figs. 4–6). These structures were almost always exposed to the tumor but remained entirely free from invasion up to the tenth day.

DISCUSSION

Mechanics of growth and expansion.—The initial necrosis which invariably followed implantation undoubtedly involved some degenerating mouse tissues included with the implant. Since it occurred in the center of the tumor rather than at the fringes, it probably did not represent a reaction between tumor tissue and the chick host. It is suggested that the bulk of the necrotic substance involved originally healthy tumor tissue which became necrotic before the tumor mass received adequate vascularization and before it shifted to a more favorable location than was afforded by the body wall.

Whereas the tumor mass was at first centered in the body wall where it was implanted, it was later found to be centered in the right mesonephros and coelom. Several possibilities suggest themselves as to the mechanism of this shifting, such as a gradual inward shift of the bulk of the tumor and an infiltration by migratory cells. After the closure of the incision wound, the median surface of the expanding tumor mass bulged into the coelom (Fig. 1), where it encountered little or no resistance. The inward shift of the lateral surface of the tumor, away from the body wall (Chart 2, Fig. 1), may have been merely passive—i.e., it may have been due to rapid increase in thickness of the host somatopleure, lateral to the tumor.

A process involving infiltration of viscera by migratory cells was suggested by the loose texture of the tumor and the amoeboid shape of many of its cells. Often isolated groups and strands of tumor cells were observed at considerable distance from the center of the tumor mass. It is likely that, when such strands of cells reached the mesonephros, they established there a vigorous local center of proliferation, which rapidly outgrew the original center. Usually a narrow cone of cells extending toward the body wall indicated the original site of the tumor (Chart 2).

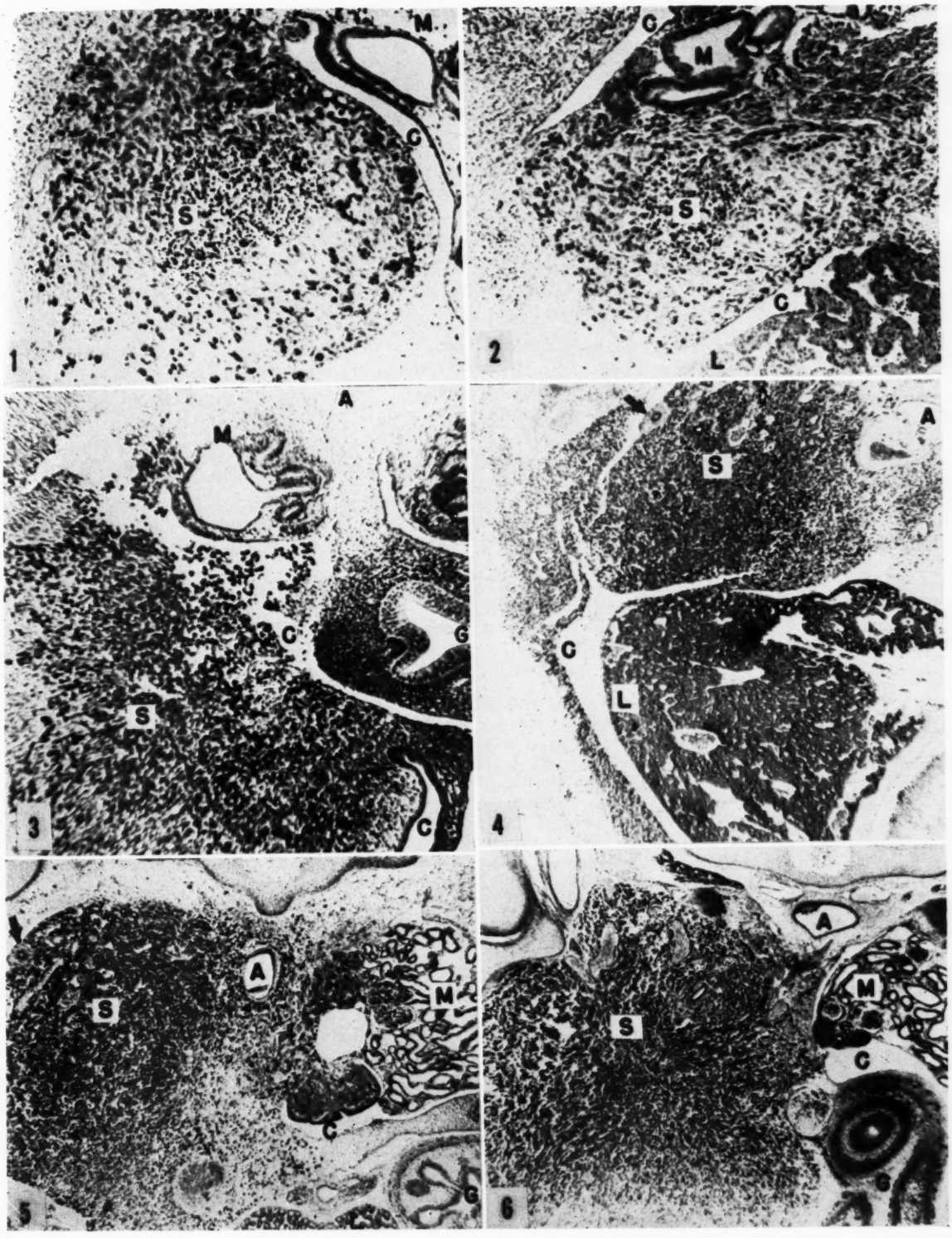
Infiltration by migratory cells can easily account for all sites of tumor invasion in our material. However, it is possible that still another mechanism of spreading occurred, especially in older cases. Isolated clusters of cells were found floating freely in the body cavity whenever the tumor mass had penetrated through the somatopleure (Fig. 3). Some of these cells may have been transported in the coelomic fluid to nearby organs, where they became lodged. On the other hand, we never observed tumor cells inside of blood vessels, and this mechanism of transport seems to be ruled out for young embryos. However, it should be remembered that our observations extended only through the ninth day of incubation.

Our observations are in accord with the evidence of Coman (3) that tumor cells are less cohesive and show a higher degree of motility than their normal counterparts. Material like that used in our investigations would be favorable for a more detailed study, *in vivo*, of these properties, since closely timed series can be obtained, and intraembryonic tumor transplants in the chick would be readily accessible to experimentation.

Selective destruction of host structures.—The problem of selective invasion by the tumor, resulting in the preferential destruction of some host tissues, while others seem to be refractory, is sharply pointed up in this study. The fact stands out that the mesonephros alone became the center of tumor growth, whereas other organs, although they may also have been invaded, did not support further expansive growth of the tumor equally well.

The following explanations of this situation suggest themselves: (*a*) the early onset of function of the mesonephros may have increased its susceptibility to invasion; (*b*) the tumor may have established itself in the mesonephros because of its high degree of vascularization; (*c*) density or mechanical resistance may have "protected" other organs; (*d*) the topographic position of the mesonephros, in

[2] Levi-Montalcini, personal communication.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All photomicrographs are of cases stained with hematoxylin.

Abbreviations: A, dorsal aorta; C, coelom; G, gut; L, liver; M, mesonephros; N, notochord; S, tumor; T, metanephros; an arrow indicates the position of the Müllerian duct.

FIG. 1.—S180-32 (5 days). Tumor with a large necrotic area in its center. The viable cells are scattered in the body wall (left); the tumor mass bulges into the coelom (right). ×65.

FIG. 2.—S180-59 (5 days). Tumor with necrotic area. A bridge spans the coelom, from body wall (left) to mesonephros (M), and a few tumor cells have invaded the interstitial spaces of the mesonephros. The adjacent liver (L) is not invaded. ×80.

FIG. 3.—S180-71 (5 days). A large tumor lies in the ventrolateral body wall. At its inner margin cells have become detached, and float freely in the coelom (C). The gut is not invaded. A few tumor cells have reached the mesonephros (M). ×70.

FIG. 4.—S180-5 (7 days). Tumor cells have infiltrated the outer wall of the coelom (left). Center of tumor (S) in the right mesonephros which has been almost completely destroyed at this level. Müllerian duct (arrow) and dorsal aorta (A) remain refractory, while the liver (L) is superficially invaded. ×30.

FIG. 5.—S37-3 (7 days). The right mesonephros has been entirely replaced by the tumor at this level, and a few peripheral cells have migrated to the left mesonephros (M). Dorsal aorta and Müllerian duct (arrow) are intact. Right gonad and adrenal are absent. ×25.

FIG. 6.—S180–125 (8 days). Cartilages of the pelvic girdle, nerves (upper left), and a sympathetic ganglion are partially imbedded in the tumor, but not invaded. The Wolffian duct (just left of "S" label) is imbedded in the tumor but remains intact. ×25.

the neighborhood of the advancing tumor, may have increased its chance of becoming a target for attack. Yet the mesonephros, invariably the most severely invaded, holds each of these properties in common with other structures, which were equally accessible to the tumor but remained partly or completely refractory. Whatever biochemical constituents of the mesonephros may have combined with the mechanical factors suggested above to favor it as a site of tumor establishment are yet to be demonstrated.

SUMMARY

Small portions of mouse Sarcomas 37 and 180 were implanted in the somatopleure of 2½-day chick embryos. The growth pattern of the tumor in the host is described. Two phases were distinguished: an initial regressive phase, which is followed by a phase of active growth. During the latter period the tumor was highly invasive of various chick tissues. The mesonephros assumed a unique position in that it was preferentially attacked and partially destroyed; it became invariably the growth center of the tumor. The metanephros, blood vessel walls, cartilage, nerves, and ganglia were completely refractory. Some other structures fell into an intermediate category, being only temporarily refractory or slightly invaded. The reasons for this selectiveness by the tumor are not yet evident, although a number of factors which may have contributed to the susceptibility of the mesonephros to tumor invasion are discussed.

ACKNOWLEDGMENTS

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Incorporation and Turnover of Radiophosphorus in Mouse Mammary Tumors (dbrB and C3H)*

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INTRODUCTION

Previous studies of two mouse mammary adenocarcinomas, histologically very similar, revealed a significant difference in their respiratory and over-all metabolic activity *in vitro* as determined by the Barcroft-Warburg manometric method (3). The tumors investigated were the mammary adenocarcinoma grown in the DBA strain of mice, designated the dbrB tumor, and that grown in the C3H strain, designated the C3H tumor. The former proved to be the faster growing one. The O_2 uptake and aerobic glycolysis of the relatively faster growing tumor were about 3 times as high as those of the slower growing one. Further investigations on the phosphorylated intermediates of these tumors were carried out by use of freezing technic and enzymatic methods (5). The components determined included total acid soluble phosphorus, inorganic orthophosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1,6-diposphate, phosphoglyceric acid, coenzyme I, adenylic acid, adenosine diphosphate, adenosine triphosphate, and phosphocreatine. Levels of lactic acid and glycogen were also measured. The more rapidly growing mammary tumor showed significantly higher levels of glucose-1-phosphate and adenosine triposphate (ATP) and a lower level of inorganic phosphorus. These findings suggested that a higher level of energy is available for vital functions in the faster growing tumor. It was of interest to investigate the dynamic metabolic behavior of these tumors *in vivo*. The results of this investigation, carried out by the use of the tracer technic with radioactive phosphorus, are reported in this communication.

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EXPERIMENTAL PROCEDURE

C3H strain mice weighing between 25 and 26 gm., bearing the C3H mammary tumor, and mice of the same weight range of the DBA strain bearing the dbrB mammary tumor were injected intraperitoneally with radio-phosphorus[1] (as inorganic orthophosphate). The dose varied between 13 and 2.5 million counts/min in a volume of 0.5 ml.–1.0 ml. corresponding to initial levels of 131–235 μc. (between 22 and 40 r) and containing 0.16 to 0.4 μM of inorganic orthophosphate. That this dose of radiation had no effect on the viability of the tumor cells is demonstrated in Figures 1 and 2, where mitotic figures can be noted. This number of counts was injected into the first animals of each group at the start of each experiment. The same quantity of phosphorus was injected into subsequent animals at later dates and the counts corrected for loss of radioactivity with time. At time intervals between ½ hour and 192 hours, the tumors were excised under nembutal anesthesia. The intact portions from the edges of the tumors were quickly dissected, homogenized, and deproteinized with ice-cold 10 per cent tricholoroacetic acid, as previously described (2) and analyzed.

Radioactivity was measured both in the protein-free trichloroacetic acid extracts and in tricholoroacetic acid residues. The latter were dissolved in hot 30 per cent sodium hydroxide before aliquots were counted.

For measurements of specific activity[2] of the inorganic and adenosine triphosphate phosphorus, the tricholoroacetic acid extracts were treated in accordance with the scheme shown in Table 1. This procedure utilizes a Dowex ion exchange resin #1 (200–400 mesh) and enables one to separate the different nucleotide-containing fractions by selective elution from the column, according to the

[1] Obtained from Oak Ridge National Laboratory, Tenn.

[2] Specific activity = counts/min/μg phosphorus.

method of Cohn and Carter (1). Since we were interested chiefly in the ATP fractions (G, H, J), no attempt was made to separate the components of the other fractions. Fractions (D, E, F) which contained almost all the inorganic phosphorus, AMP, and ADP (the latter two in comparatively small amounts) were used for determining the specific activity of inorganic orthophosphate. Proof that fractions G, H, J were analytically ATP was obtained from spectrophotometric measurements of adenine and chemical determinations of organic phosphorus. The recoveries for extracts of tumors in terms of radioactivity after passage through the column are shown for a typical experiment in Table 2.

TABLE 1

PROCEDURE OF FRACTIONATION

Tumor

homogenize in 10 per cent TCA; centrifuge; re-extract 2× in 5 per cent TCA

ppt. dissolve in 30 per cent NaOH (II)

combine supernatants; neutralize (I); add 3 vol. alcohol and BaAc

Ba ppt. dissolve in HCl and H_2O; Ba precipitate out with Na_2SO_4; neutralize; make 1M with respect to NH_4OH. Initial solution (A)

collect supernatant and washings (III)

Run through Dowex column 3×
Solution not taken up by resin (B)
Wash resin with water (C)
Wash resin 3 × with 0.02 M NaCl in 0.01 M HCl; (D)
(this fraction contains adenine, (E)
adenosine, AMP, ADP, and inorganic P) (F)
Wash resin 3 × with 0.2 M NaCl in 0.01 M HCl; (G)
(this fraction contains ATP) (H)
(J)

RESULTS AND DISCUSSION

Table 3 summarizes the data on the radioactivity of the trichloroacetic acid extracts from C3H and dbrB mammary tumors at varying time intervals following injection of radiophosphorus. The figures represent the percentage of the total dose injected which is present in actively growing intact portions of the tumors. It should be noted that the total number of counts taken up is less for the C3H tumor than for the dbrB, that for both tumors the maximum uptake has already occurred by the end of ½ hour, and that the C3H tumor loses its radioactive phosphorus from this fraction much more slowly than does the dbrB tumor, having a "half-life" of 32 hours, as compared to 18 hours. These figures were obtained by graphic means from the data in the table.

Table 4 summarizes a typical experiment carried out with both types of tumors (dbrB and C3H), in which the animals were sacrificed 1 hour after injection of radiophosphorus. In this experiment fraction A (see Table 1) was passed through the resin so that the specific activity of the fractions containing inorganic and ATP phosphorus could be measured. As shown above, less phosphorus is taken up in the TCA extract and residue of the C3H tumor than in those of the

TABLE 2

RECOVERIES IN RADIOACTIVITY AFTER FRACTIONATION PROCEDURE

Total number of counts in TCA extract	466,000 counts/min
Counts in III (Table 1)	23,600
Total counts in fraction A put through resin	457,000
Counts in B	4,000
C	280
D	1,150
E	196,000
F	122,000
G	79,800
H	26,400
J	16,200
Total counts recovered from resin	445,830
Per cent recovery	97

TABLE 3

RADIOACTIVITY IN TCA FRACTION/GM TUMOR AS PER CENT OF TOTAL COUNTS/MIN INJECTED

	C3H	dbrB
½ hour	1.77 (2)*	2.71 (3)
1	1.70 (2)	2.52 (6)
2	1.51 (1)	1.98 (2)
24	0.99 (2)	1.19 (3)
48	0.77 (1)	0.66 (2)
96	0.47 (2)	0.73 (1)
120		0.53 (1)
148		0.34 (1)
192	0.26 (1)	
half radioactivity	0.89	1.35
time to lose half radioactivity	32 hours	18 hours

* Number of experiments.

dbrB tumor. It should also be noted, in accordance with our earlier studies (5), that the C3H tumors have less ATP (here expressed as adenine) than the dbrB. That the fraction which we call ATP agrees analytically with expectations is also shown (theoretical ratio adenine: organic P = 1.00:3.00). The specific activities of the phosphorus in ATP in both tumors are also presented in the table. It is clear that, although the phosphorus in the ATP of the dbrB tumors is completely equilibrated with the inorganic phosphorus at the end of 1 hour, the ATP phosphorus of the C3H tumor is only 70 per cent equilibrated.

An experiment similar to the one just described, but in which the animals were sacrificed 96 hours

after injection of radiophosphorus, is shown in Table 5. In this experiment, only 13,512,000 counts/min were injected. However, in the table the values are corrected for an injection of 24,270,000 counts/min, so that the data can be compared to those of the preceding table. It should be noted that, whereas the specific activity of the ATP phosphorus in both tumors has gone down, the loss in the dbrB tumor is greater than that shown by the C3H tumor, indicating a more rapid turnover in the dbrB tumor. This is also true of the radioactivity lost from the TCA extracts, as pointed out earlier. Furthermore, after 96 hours the radioactivity of the entire dbrB tumor (TCA extract and TCA residue) was 23 per cent less than it was after 1 hour, while that of the C3H tumor was 37 per cent greater. This again points to a more rapid turnover of radiosphorus in the metabolically more active dbrB tumor.

The experiments just reported confirm our earlier conclusions about the C3H and dbrB tumors. These conclusions were based on studies of metabolic rates (3), mitotic indices (4), latent periods (2), and static levels of the phosphorylated intermediates (5). The present work has proved that the dbrB tumor takes up more radiophosphorus at a more rapid rate, not only into those constituents of the tumor which are TCA-soluble, but also into those which form the TCA residue (nucleoproteins, phospholipids, and proteins). The dbrB tumor also loses the radiophosphorus from these fractions more rapidly than does the C3H tumor, indicating more rapid turnover in the former, an additional indication of higher metabolic activity.

TABLE 4

ACTIVITY OF VARIOUS FRACTIONS OF C3H AND dbrB TUMORS REMOVED 1 HOUR AFTER INJECTION OF RADIOPHOSPHORUS

	C3H	dbrB
Age of tumor	20 days	13 days
Wt. of tumor	1.15 gm.	0.90 gm.
Cts. injected	24,270,000	24,270,000
Cts. taken up by tumor in TCA extract/gm tissue	314,000 counts/min	679,500 counts/min
Cts. taken up in TCA residue/gm tissue	43,900 counts/min	106,200 counts/min
ATP-adenine	165 μg/gm	226 μg/gm
Molar ratio of adenine/P in ATP	1.00:3.09	1.00:308
Specific activity* ATP phosphorus	556	1161
Specific activity Inorg. P	786	1140
Per cent equilibration	70.3	101.7

* Specific activity = counts/min/μg P.

TABLE 5

ACTIVITY OF VARIOUS FRACTIONS OF C3H AND dbrB TUMORS REMOVED 96 HOURS AFTER INJECTION OF RADIOPHOSPHORUS

	C3H	dbrB
Wt. of tumor	0.75 gm.	1.52 gm.
Cts. remaining in TCA soluble extract/gm tissue	115,000 counts/min	178,000 counts/min
Cts. remaining in TCA residue/gm tissue	175	243
Molar ratio of adenine/P in ATP	1.00:3.31	1.00:2.97
Specific activity ATP phosphorus	217	282
Specific activity Inorg. P	217	255
Radioactivity lost from TCA extract, as per cent of total taken in between 1 and 96 hours	63.4	73.8
Per cent loss in specific activity of ATP phosphorus between 1 and 96 hours	61.0	85.5
Change in radioactivity in total TCA acid soluble and insoluble fractions, as per cent of radioactivity present at 1 hour	+37	−23

To rule out the possibility that the different amounts of radioactive phosphorus taken up by the tumors were due to different levels of radioactive phosphorus in the plasma, the following experiment was carried out:

DBA and C3H mice, weighing 28 gm. each, were injected intraperitoneally with 0.5 cc. of radioactive phosphorus containing 18,300,000 counts/min, equivalent to 178 μc. and containing 0.4 μM of phosphorus. At the end of 1 hour, the mice were anesthetized with nembutal and blood removed directly from the heart under heparin. Blood from three animals was combined and the plasma separated by centrifugation. The plasma was deproteinized with 10 volumes of 5 per cent

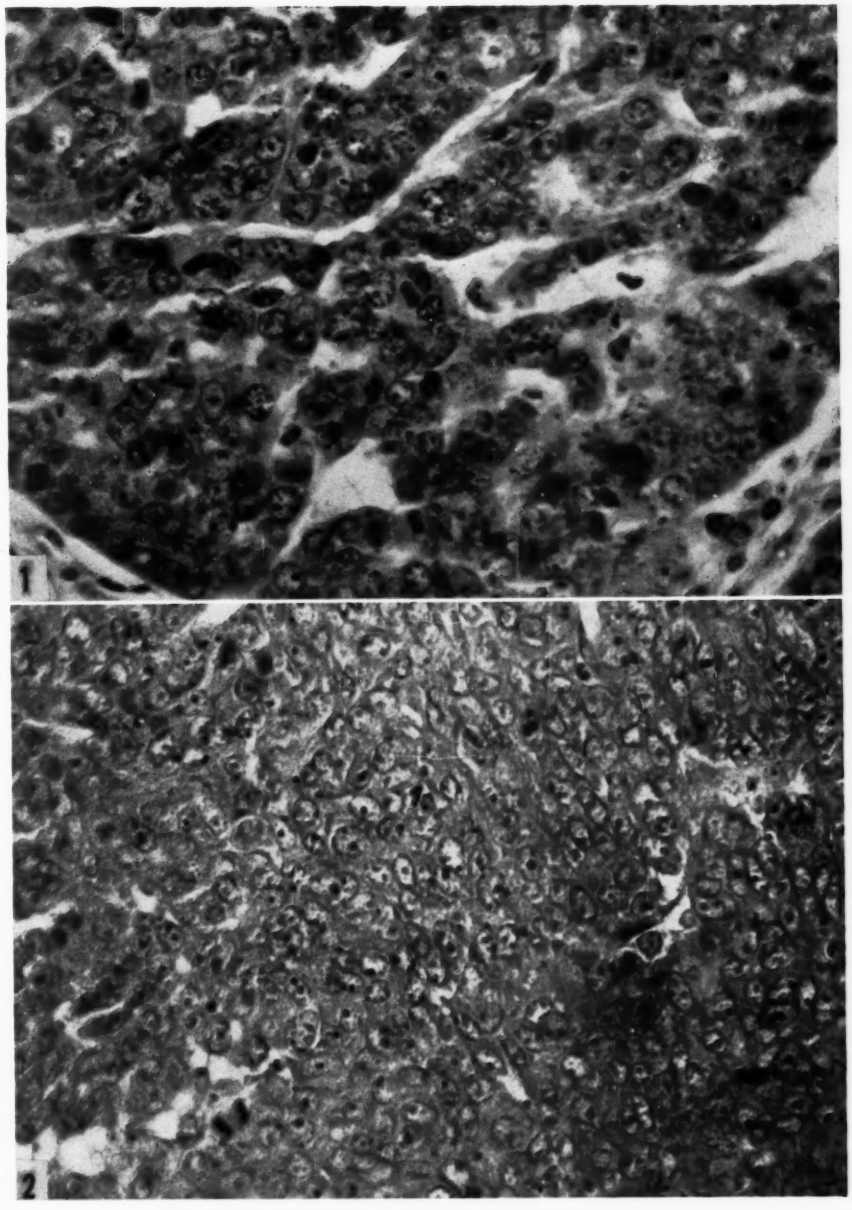

FIG. 1.—DbrB mammary tumor removed from DBA mouse 1 hour following injection of P^{32}, dose 175 μc. Note numerous mitotic figures. ×450.

FIG. 2.—C3H mammary tumor removed from a C3H mouse 1 hour following injection of P^{32}, dose 174 μc. ×450.

trichloroacetic acid. Aliquots of this protein-free filtrate were assayed for radioactivity and inorganic phosphorus. The inorganic phosphorus content of the plasma for both strains of animals was 11.3 mg. per cent. The plasma of the C3H mice at the end of this time interval contained 205,400 counts/min/cc of plasma: the plasma of the DBA mice contained 204,800 counts/min/cc of plasma. It is clear from these results that the different amounts of radiophosphorus taken up by the tumors in the two strains of animals cannot be attributed to differences in plasma level.

SUMMARY

Studies have been carried out on the incorporation and turnover of radiophosphorus into various fractions derived from the C3H and dbrB mouse mammary tumors, grown in the respective strains of mice. The results indicate again that the dbrB tumor has a higher rate of metabolic activity than the C3H tumor and substantiate those obtained from previous *in vitro* studies. It is concluded that these mouse mammary adenocarcinomas, although morphologically similar, exhibit significant differences in their physiological behavior.

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Progressive Microscopic Alterations in the Livers of Rats Fed the Hepatic Carcinogens 3′-Methyl-4-dimethylaminoazobenzene and 4′-Fluoro-4-dimethylaminoazobenzene*

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The histology of the livers of rats fed 4-dimethylaminoazobenzene (DAB) and of the hepatic tumors induced by this dye has been studied in detail (5, 6, 14, 16). Studies of the tumors produced by other aminoazo dyes have indicated that these neoplasms are morphologically similar to those induced by 4-dimethylaminoazobenzene (3, 12, 22). There have been some recent observations, however, which indicate that the sequences of histological changes that parallel the development of these tumors may differ. For example, it was noted that the ingestion of the very active carcinogen 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) for only 4 weeks produced a marked increase in the number of bile duct-like cells in the liver; this change was not observed when 4-dimethylaminoazobenzene was fed for the same period of time (19, 20). The 3′-methyl derivative also appeared to be more active in the production of gross cirrhosis than was 4-dimethylaminoazobenzene (11) and, when fed at equivalent levels in the diet, had considerably more effect in altering the intracellular composition of rat liver (19, 20). However, 4′-fluoro-4-dimethylaminoazobenzene (4′-F-DAB), which is about as potent a carcinogen as 3′-Me-DAB (12), did not exert nearly so great an effect on the intracellular composition of the livers of rats fed the dye (20).

In view of these observations the livers of rats fed the 3′-methyl and 4′-fluoro derivatives of 4-dimethylaminoazobenzene have now been studied histologically at frequent intervals from the beginning of dye feeding up to the emergence of gross tumors. In these studies an effort has been made to correlate the histological changes in the livers with the levels of protein-bound aminoazo dyes, since the changes in the bound dye content of the livers of rats fed various derivatives of 4-dimethylaminoazobenzene have been correlated with the carcinogenic potencies of these dyes (10). The tumors produced by the two carcinogens have been compared microscopically, and an attempt has been made to determine the site of origin of the neoplasms. In addition, studies have been made of the reversibility of some of the preneoplastic pathological changes by withdrawing the carcinogen from the diet after pronounced alterations in the liver had occurred. Finally, all these observations have been considered in relation to the problem of the pathogenesis of the liver carcinomas.

METHODS

A preliminary experiment was conducted with 3′-Me-DAB to determine the times at which the early changes, such as bile duct proliferation, occurred. Male rats[1] weighing 190–205 gm. were fed ad libitum a semi-synthetic diet ([9], cf. diet 3), modified to contain 24 per cent of casein by the addition of sufficient low-vitamin casein[2] at the expense of the glucose. This change was made because the bile duct hyperplasia was more extensive at 4 weeks when the ration contained 24 per cent rather than 12 per cent of casein (20). The modified diet consisted of casein, 24; rice bran concentrate (Vitab), 2; salts, 4; glucose monohydrate (Cerelose), 65; corn oil, 5; halibut liver oil, 0.03; and 3′-Me-DAB, 0.064. The riboflavin content of this diet was 0.8 mg/kg. Animals were killed in groups of two with ether after 1, 2, 4, 6, 8, 11, 14, 18, 21, 25, 28, 32, 42, and 58 days, and similar groups of animals on the same diet without the carcinogen were killed after 0, 6, 14, 25, 28, and 58 days as controls. The liver, spleen, kidneys, pancreas, lungs, thyroid, adrenals, duodenum, common bile duct, and bladder were removed and

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[1] Obtained from the Holtzman-Rolfsmeyer Rat Company, Madison, Wis.

[2] "Vitamin-Free" Casein, Nutritional Biochemicals Corp., Cleveland, Ohio; this preparation contained about 0.6 μg. riboflavin per gram.

pieces fixed in 4 per cent formaldehyde, Bouin's fluid, and Zenker's fluid. The tissues were subsequently imbedded in paraffin, sectioned, and stained with hematoxylin and eosin. This preliminary experiment indicated that no pertinent changes occurred in any organs other than the liver and spleen.

The second series was studied in a similar fashion, except that a third group of animals was fed 4′-F-DAB. Both carcinogens were fed at a level of 0.058 per cent of the diet, or 90 per cent of the level used in the first series, to insure that an adequate number of animals survived until neoplasms developed. The riboflavin content of the diet was also increased to 1.5 mg/kg for the same reason.

In this series three or more animals fed each dye were killed at 7, 14, 20, 24, 28, 32, 35, 40, 44, 49, 56, 63, 77, 91, 112, 119, and 133 days. Additional animals fed 3′-Me-DAB were killed in groups of three at 11, 16, 18, 22, 26, and 30 days. During the first 9 weeks representative animals were selected on the basis of their weights. At 90 and 111 days laparotomies were performed on the survivors to select the animals with tumors, since these neoplasms are not suitable for microscopic study when they become palpable. When gross tumors were observed, sections were invariably prepared from them in order to examine the histological pattern and malignancy of the neoplasm. Control animals were killed in groups of three at 0, 28, 56, and 133 days. A group of animals fed 3′-Me-DAB for 35 days was transferred to the same diet without the carcinogen, and these rats were killed in groups of three after 5, 14, 21, 28, 42, 56, and 98 days on the basal diet.

In the second series the animals were killed with ether, the livers immediately exposed in a cold room (0–5° C.), and either the right, the left, or the median lobe ligated close to the vena cava. The unligated lobes were perfused with cold (0–5° C.) 0.14 M NaCl. Since three animals were used each time, the nonperfused lobe was alternately the right, left, and median in order to obviate possible unequal lobar effects by the carcinogens. The liver was excised, slices from the separate lobes were placed in the fixatives used previously, and sections were prepared for histological study. The remainder of the perfused lobes was homogenized in water, and the crude protein was isolated and analyzed for protein-bound dye (8, 10).

The histological procedures included technics which require further description. As a routine, the sections were stained with hematoxylin and eosin. This was varied by a phloxine-methylene blue-azure blue technic, by Mallory's phosphotungstic acid hematoxylin method, and by Regaud's stain (4). For the demonstration of mitochondria, sections of formalin-fixed tissues were postchromated after deparaffinization by the technic of Mullen and MacCarter (13). Bullard (1) has reported that this procedure is satisfactory for the staining of mitochondria. Counts of the mitotic figures were made on sections from tissue fixed in Bouin's fluid, since these sections permitted clear definition of the mitotic figures. Twenty-five high-power fields (4 mm. objective, 10 × ocular) were examined around both the center and the periphery of the lobules separately. The number of mitoses observed was calculated as the occurrence per 100 fields and designated as the mitotic index.

RESULTS

General condition of the animals.—The animals fed 3′-Me-DAB in the first series had a sustained weight loss. Three of the nine animals left on the experiment died between 28 and 32 days, and autopsies indicated that death was probably due to hepatic insufficiency (see below). There was a moderate amount of ascitic fluid and considerable edema of the pancreas in both animals killed at 21 and 25 days and in one of the two killed on the 28th day, but these conditions were not observed subsequently. Ascities was not seen in the control animals of either series or in the dye-fed animals of the second series.

In the second series the animals fed either dye lost weight during the first 3 or 4 weeks, regained their starting weights between the fourth and sixth weeks, and thereafter continued to gain slowly. All the animals fed 4′-F-DAB survived the desired length of time. Between the third and fifth weeks, five of the animals fed 3′-Me-DAB were destroyed because of respiratory infections, three were discarded when moribund, and two were lost by cannibalism.

Gross pathology.—In the first series the earliest hepatic changes appeared at 18 days and consisted of pallor and a fine granularity of the surface. This was more conspicuous at 21 days, at which time the ascites and pancreatic edema were also seen. The granularity increased gradually, and by 42 days the livers appeared severely cirrhotic. In the second series similar hepatic changes were produced by both carcinogens, but they began about 1 week later. At laparotomy on the 90th day three of seventeen survivors fed 3′-Me-DAB and six of nineteen fed 4′-F-DAB had small tumors. All the animals fed either dye had moderate to severe hepatic cirrhosis. No tumors were found earlier in either group. At 111 days nine of fourteen animals fed 3′-Me-DAB and ten of thirteen fed 4′-F-DAB had gross tumors and were autopsied within a

week. At 133 days all the survivors had tumors. Frequently, a few patches of pneumonia were seen in the lungs, and the spleens were larger and darker in color than normal. No gross evidence of disease was observed in other organs. No metastases were found except for one tumor nodule implanted in a laparotomy scar.

Microscopic pathology.—The earliest microscopic change in the livers was seen after 7 days of ingestion of the carcinogen, when small hyaline droplets appeared in the cytoplasm of some of the parenchymal cells. By 11 and 14 days they were found in the cells of most liver lobules. As their size increased they assumed the shape of a sausage or horseshoe, and eventually some of these hyaline inclusions encircled the nuclei. Usually a free rim of apparently normal cytoplasm intervened between the inclusion and both the cell membrane and the nucleus. The hyaline, eosinophilic appearance of the inclusions contrasted sharply with the granularity of the normal liver cytoplasm (Fig. 1).

An interesting feature of this early cytoplasmic change was its distribution. When 3′-Me-DAB was fed, the inclusions were seen first in the periphery of the lobule (i.e., about the portal areas). As they increased in number, they were progressively noted in cells closer to the central (hepatic) veins. When 4′-F-DAB was fed, the first hyaline droplets and inclusions appeared about the central veins. As their number increased, they were progressively noted in more peripheral cells, until by 20 days cells at the periphery of the lobule were also involved. One could determine by this difference alone which carcinogen the animals had ingested until subsequent alterations in the lobules made identification of central and portal veins difficult. Neither the type of fixative nor the perfusion affected the appearance of the inclusions. On reexamination of sections from a previous study,[3] this cytological change was found in the peripheral cells of lobules from the livers of rats fed 4-dimethylaminoazobenzene.

The second change in the livers of rats fed 3′-Me-DAB was the increased prominence and cytoplasmic basophilia of the bile ducts; this occurred between the eleventh and fourteenth days. A similar change was noted at about 20 days in the livers of rats fed 4′-F-DAB. At 18–24 days a slight increase occurred in the number of bile duct cells in the livers of rats fed the 3′-methyl derivative (Fig. 2). A rapid increase in the numbers of these cells followed until, by 26 to 28 days, they had penetrated close to the central veins of most lobules (Figs. 3 and 4). The parenchymal cells vanished as the proliferating bile duct cells increased; very little distortion of the lobular pattern occurred. Most cells immediately adjacent to the bile duct cells contained large hyaline inclusions, and pyknosis, karyorrhexis, and karyolysis of hepatic nuclei were conspicuous in these areas. Many of the duct cells were arranged in a glandular pattern in which many lumina were evident (Fig. 5). In other areas the duct cells grew to form cords and sheets. Some connective tissue was seen in these regions, and numerous small capillaries were present. This appearance was maintained for only a short time; for at approximately 30 days (25 days in the first series) the epithelium became more basophilic and columnar, and the lumina in some of the ducts became larger (Fig. 6). The cells of these large ducts contained many mitotic figures, and the lumina were filled with polymorphonuclear leukocytes and eosinophilic coagulum. Such areas have been regarded as adenomas and persisted until malignant neoplasms appeared. They were gradually surrounded by dense collagenous connective tissue and might be called "cholangiofibrosis" (Figs. 7, 16) (14). In the livers of animals placed on the basal diet after 35 days of ingestion of 3′-Me-DAB, similar areas persisted unchanged for the duration of the experiment, while the other histological changes, except for a few cysts, regressed.

On the other hand, most of the newly formed bile duct cells apparently had a different fate. At 35 days focal areas of cells with amphophilic cytoplasm appeared among the ducts. Such foci were first seen in the periphery of the original lobule among the new ducts. Although few mitoses were seen, these foci later expanded and replaced all the newly formed duct cells except those in the adenomas. This transformation also occurred in the livers of animals placed on the basal diet after 35 days of dye ingestion (Fig. 8). Where residual parenchymal cells persisted around the central veins, a thin wall of fibroblasts separated these residual cells from the new parenchymal cells (Fig. 9). Similarly, these nodules of enlarging amphophilic cells were separated from one another by thin connective tissue septa, in which more collagen was later deposited. Because of this nodular replacement of proliferated bile duct cells by cells scarcely distinguishable from the original liver cells, there was considerable distortion of the normal liver architecture, and after 7 or 8 weeks the organ was mainly a collection of nodules. Between the nodules were fibrous strands containing a few residual bile duct cells, adenomata, macrophages containing yellow pigment, and accumulations of lymphocytes and polymorphonuclear leukocytes

[3] H. P. Rusch, D. M. Angevine, and J. M. Price, unpublished data.

(Fig. 16). Some nodules of proliferating parenchymal cells (Fig. 15) also arose from the residual cells around the central vein; these were found as early as 30 days when 3′-Me-DAB was fed. These nodules contained numerous mitotic figures, were sharply circumscribed and well differentiated, and contributed to the destruction of the hepatic architecture. Few of the nodules reached a great size, probably because there was soon sufficient functioning parenchyma to cause the cessation of regenerative processes.

Extreme increases in the number of bile duct cells were found in the livers of the animals which died or were killed when moribund, while ingesting 3′-Me-DAB; no hyaline inclusions were found in these livers. In some cases many of the cells among the bile duct tissue resembled small parenchymal cells. In no instance did an animal die after 6–7 weeks when the number of parenchymal cells was greatly increased. It appears, therefore, that the bile duct cells and the new parenchymal cells among them were incapable of supporting vital hepatic functions, and hepatic insufficiency resulted. The autopsies revealed no other cause of death.

Although there was a prominence of the bile ducts in livers of rats fed 4′-F-DAB for only 20 days, a dramatic proliferation of these cells did not occur. The increase in number of these cells was moderate and reached a maximum at about 40 days (Fig. 14). These cells apparently had the same fate as those in the livers of the rats fed the other carcinogen. However, the replacement by liver cells appeared to keep pace with the rate of formation, so that no accumulation resulted. The liver architecture was destroyed, nonetheless, and after 49 days it was difficult or impossible to tell histologically which carcinogen had been fed. The adenomas appeared later when this carcinogen was fed, but, once they appeared, could not be distinguished from those produced by 3′-Me-DAB. The fluoro compound also produced necrosis around the central veins among the cells containing inclusions. Central necrosis was first seen at 20 days and was very definite after 24 days (Fig. 13). It never became much worse, and the distortion of the lobular pattern by 7–8 weeks made it difficult to determine what had happened to these areas of central necrosis.

The extensive increase in the number of bile duct cells is a real proliferation, accompanied by large numbers of mitotic figures. The average liver did not lose cells, although a decrease in liver weight occurred during the early period of ingestion of 3′-Me-DAB. With methods previously described (20), counts (7)[4] were made of the nuclei in representative samples of livers from rats fed the ring-methyl dye in the second series. The normal control livers contained an average of 156 million nuclei per gram, or about 2,070 million nuclei per liver. The number of nuclei per gram increased so that, between 16 and 26 days of ingestion of the carcinogen, the number per gram was between 220 and 229 million, and the number in the entire organ was between 1,880 and 2,260 million. At 28 and 32 days, when the number of bile duct cells significantly increased in the sections (Figs. 4, 5), the number of nuclei increased to 329 million per gram, and 3,200 million per average liver at 28 days; at 32 days the corresponding figures were 315 and 2,900 millions, respectively. There was, therefore, a real increase in cells with a marked relative and real increase in bile duct cells.

The residual parenchymal cells around the central veins contained more mitochondria than either the bile duct cells or the cells replacing the duct cells at the periphery of the lobule (Figs. 10–12). However, since it was previously demonstrated that the residual parenchymal cells around the central vein contained fewer mitochondria than normal liver cells (20), each of these cell types must contribute to the alterations in intracellular composition of the livers of rats fed 3′-Me-DAB (19, 20). The present results again emphasize that biochemical studies on isolated cellular fractions may be advantageously accompanied by microscopic studies of the intact cells and tissues. These studies help to differentiate between alterations in cellular biochemistry and changes in cell type.

Mitctic counts in relation to cytological changes.—In the livers of the animals fed 3′-Me-DAB a sudden increase in the number of mitotic figures occurred at about the sixteenth day (Chart 1). This increase was largely in the peripheral parenchymal cells but was paralleled by a simultaneous but much slighter mitotic activity in the centrilobular parenchymal cells. This augmented mitotic rate, which immediately preceded the peak of the cytoplasmic inclusion formation, was suppressed at the time of necrosis and degeneration of the peripheral cells around the 25th day. At about this time the tremendous increase in bile duct cells occurred and was associated with a similar great mitotic activity (Chart 1) which persisted until the ducts reached their maximal expansion in a few days. A considerable increase in mitoses among the centrilobular cells at the time of the greatest duct proliferation was also apparent. Although the mitotic rate then decreased somewhat, it was still maintained at a

[4] We are indebted to Dr. A. K. Laird for these determinations.

relatively high level; a terminal increase accompanied definite adenomatous formation after 45 days. The mitotic rate in the livers of rats fed the halogen derivative manifested a slower and less exaggerated rise (Chart 2), which was proportional in rate and magnitude to the simultaneous increase in bile duct cells. These mitotic figures were typical and normal. Abnormal figures were not seen until malignant neoplasia occurred. It is significant that during the period when the newly formed duct cells were being replaced by or transformed into parenchymal cells there was a depression in the rate of mitosis.

Neoplasms found.—The malignancies that were produced were classified simply as carcinoma of the liver, although, in conformity with the usual classification, they have been subdivided for purposes of illustration into "hepatomas," "cholangiomas," and "mixed tumors." The "hepatomas" tended to reproduce the histological appearance of liver parenchyma (Fig. 19). In general, the cells tended to invade the adjacent tissue, including the

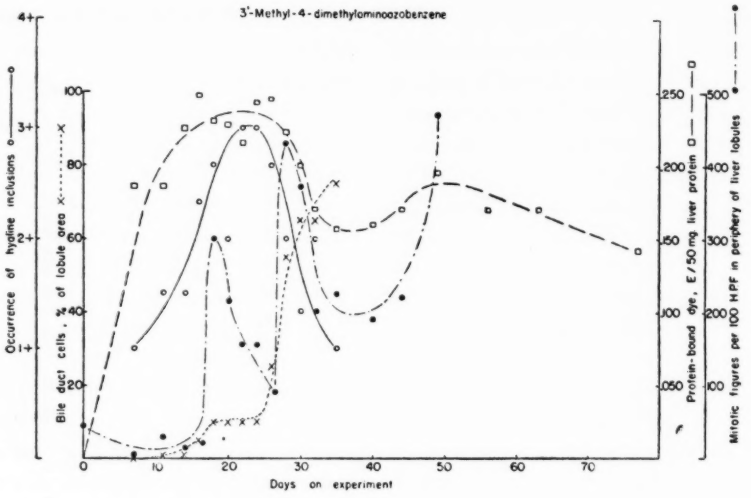

CHART 1

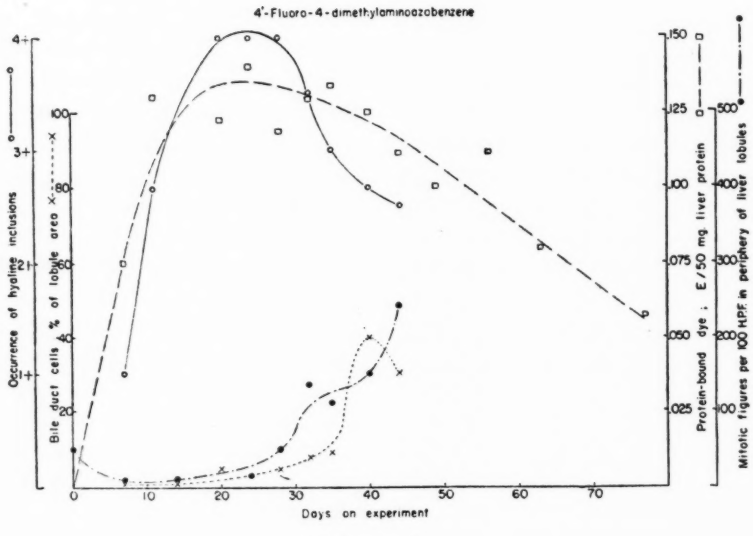

CHART 2

CHARTS 1 AND 2.—Levels of total protein-bound dye in the livers compared with certain microscopic alterations in the tissue. Protein-bound dye is expressed as E/50 mg of protein (8). These values may be converted to micromoles (10), but the shape of the curve would be the same. The hyaline inclusions are rated as follows: 1+, just detectable; 2+, readily found in most lobules but not present in large numbers; 3+, moderate numbers of inclusions; 4+, inclusions were so abundant as to be present at the opposite extreme of the lobule from that in which they were first seen. The estimations of the hyaline inclusions and bile duct cells are averages of the results of two observers, each of whom examined all the sections that were made.

blood vessels. They were basophilic, had numerous atypical mitotic figures, and an increased nucleus/cytoplasm ratio. As Opie (14) has pointed out, the hepatomas resemble areas of focal hyperplasia of the parenchymal cells. However, the areas of focal hyperplasia usually had more nearly normal mitotic figures and histological arrangement, and they rarely showed any evidence of inflammation or increase in the nucleus/cytoplasm ratio. The "cholangiomas" had the appearance of an adenocarcinoma, with glandular and ductlike structures (Figs. 17, 20). The cells of the ducts were often several layers thick with evident loss of polarity. There was a marked inflammatory reaction in and around the tumors; this was especially evident in the larger ducts. Atypical mitosis, increased nucleus/cytoplasm ratio, and deep basophilia also characterized these tumors. The mixed tumors were often the larger ones and presented areas characteristic of hepatomas and cholangiomas, often with blending of one type into the other, such as that illustrated in Figure 18.

One can distinguish between the "malignant cholangiomas" and the benign bile duct adenomas (or the "cholangiofibrosis"). Both had abundant, dense, connective tissue stroma. The duct cells in the adenomas were deeply basophilic and had numerous normal mitotic figures; there was usually a single layer of cells lining the ducts, and the cells retained their polarity. After malignancies appeared, the "cholangiomas" were found to be associated with adenomas or cholangiofibrosis in more than two-thirds of the cases.

In the livers of the 19 animals fed 4′-F-DAB for over 90 days a total of 31 tumors were studied. Fifty-six malignancies from the 17 rats fed the ring-methyl derivative for the same period of time were studied. When it became evident that so many cholangiomas were associated with cholangiofibrosis (bile duct adenomas), all malignancies were carefully examined to determine whether it was doubtful, possible, probable, or certain that they were associated with such areas. Table 1 is a summary of these observations. These results indicated the tendency of all the malignancies, but especially the cholangiomas and mixed tumors, to arise from areas of bile duct adenomas. If serial sections had been made, more of these tumors might have been found to have an origin in areas of cholangiofibrosis.

Relationship between pathological changes and protein-bound dye curves.—Chart 1 shows the relationships between the level of protein-bound dye, the number of hyaline inclusions, and the bile duct hyperplasia in the livers of rats fed 3′-Me-DAB. It is evident that the protein-bound dye curve is similar in shape to the hyaline inclusion curve. As the proliferation of bile duct cells reached a significant degree, both the level of protein-bound dye and the number of hyaline inclusions decreased. In Chart 2 a similar correlation may be found for the livers of rats fed 4′-F-DAB. There was less bile duct proliferation in these livers, and the bound dye curve had a more gradual downward course than that seen when 3′-Me-DAB was fed. This leads one to correlate the increase in new bile duct cells with the decrease in protein-bound dye; their appearance in large numbers at about 26–32 days may have contributed to the fall in the level of the protein-bound dye. The second peak in the bound dye curve at 49 days in Chart 1 was probably significant. If the highest value at 49 days was considered to be in error, there are 4 other points above the value found at 35 days. A previously published bound dye curve for this carcinogen revealed a tendency to assume the shape of the one in Chart 1 (9), although the points were insufficient to establish a second peak. This second peak coincided with the replacement of the bile duct cells by cells resembling parenchymal cells and was actually anticipated from the microscopic studies before determinations of the bound dye had been made. The less dramatic appearance and replacement of bile duct cells when the halogen derivative was fed led to a prediction that a prominent second peak would not appear in the bound

TABLE 1

RELATIONSHIP OF TUMOR ORIGIN TO CHOLANGIOFIBROSIS

Dye fed	Type of tumor	Total no.	Per cent of total tumors	Number associated with cholangiofibrosis			
				Doubtful	Possible	Probable	Certain
3′-Me-DAB	Hepatoma	25	45	3	9	3	10
	Cholangioma	19	34	1	2	4	12
	Mixed	12	21	0	1	5	6
	Total	56		4	12	12	28
4′-F-DAB	Hepatoma	16	52	4	4	3	5
	Cholangioma	6	19	0	2	1	3
	Mixed	9	29	0	1	4	4
	Total	31		4	7	8	12

dye curve, and Chart 2 shows that this prediction was correct.

In the first series protein-bound dye was not measured, but the curves for hyaline inclusions and bile duct cells were similar to those presented in Chart 1, except that both curves reached maximum levels earlier. The curves for hyaline inclusions, bile duct hyperplasia, and mitotic figures were not plotted for later stages because of the profound distortion of the liver architecture.

DISCUSSION

The earliest significant histological alteration which appeared in the livers of animals fed either of these carcinogens was the development of hyaline inclusions in the cytoplasm of the parenchymal cells. They appeared the same, irrespective of the carcinogen used, although the site within the lobule was influenced by the dye fed. The origin and composition of these hyaline inclusions were not elucidated by the present study. Their staining characteristics and histochemical properties were different from those of the mitochondria, and there was no definite evidence that they were derived from any one cytoplasmic component. It is noteworthy that the variations in the number of inclusions and the amount of bound dye were parallel. Therefore, although there is no evidence to support the hypothesis that the inclusions are morphological manifestations of the protein-bound aminoazo dye, the close manner in which the changes in the dye levels follow the alterations in the inclusion counts might favor this relationship. The inclusions are probably broken by homogenization of the liver, since particles resembling these bodies have never been observed in any cell fraction which was isolated from a homogenate by centrifugation.[5] If so, the protein of these inclusions would probably appear in the supernatant fluid (nonsedimented) fraction of the liver cells. This fraction has also been shown to contain the majority of the protein-bound dyes (18–21). These inclusions are different from the cytoplasmic inclusions seen in regenerating rat liver (17).

It is not certain that the inclusions are a degenerative change, although they were followed by a necrosis in the region of their most intense aggregation, either peripheral or centrilobular. The zone of necrosis and the majority of the cells containing inclusions were rapidly replaced by an extensive hyperplasia of bile ducts when 3′-Me-DAB was fed which within a few days obliterated more than two-thirds of the lobules in some cases. This was a critical period, and survival was in part probably determined by the amount of residual parenchyma and its functional capacity.

When proliferation of ducts had reached a maximum and the animal survived the critical period, a regression of the bile duct cells usually occurred. The residual parenchymal cells remained as situated, with some extension or increase, and were surrounded by a thin ring of fibroblasts. The duct cells were rapidly replaced by parenchymal cells which, however, contained only a few mitochondria, as compared to the residual parenchyma. With further differentiation, these cells were indistinguishable from the original parenchymal cells. The pathogenesis of this duct proliferation appeared to include a direct stimulation of the duct cells by the carcinogen or a metabolite of it, and a noxious action on the adjacent parenchymal cells, which were then displaced by the proliferant duct tissue. It is pertinent that with the halogenated dye the proliferating duct cells did not penetrate into the lobule until the cells at the periphery became filled with inclusions. This may in part account for the slower onset of the duct hyperplasia and the relatively greater concentrations of inclusions found with the halogenated derivative.

It was pointed out that the rapid appearance of duct proliferation corresponded chronologically to some depletion of bound dye. Another aspect of interest was the prediction from the cytological evidence that a second peak of the bound dye curve might be expected in animals fed 3′-Me-DAB. If the new duct cells were metabolically inert in binding the dye, it might be expected that, as they were replaced by parenchymal cells, the capacity of the liver to bind the dye would attain a new peak—in the present series, at about 45–49 days. Analyses revealed such a peak at the expected time. With 4′-F-DAB the gradual increase and decrease of duct tissue precluded the establishment of a second peak.

The abrupt replacement of the duct cells by parenchymal tissue which eventually assumes normal histologic appearance poses the problem of the origin of this new generation of parenchymal cells. Because of the low mitotic index, their low content of mitochondria, their nuclear characteristics, and some tendency to occur around lumina, it is probable that they are derived by conversion from the hyperplastic duct cells. Kinosita (6) suggested a similar transition of cell-type in the livers of rats fed DAB. Such a transformation could explain the source of the adenomatous hepatomas, which

[5] The livers from rats fed 3′-Me-DAB and 4′-F-DAB for studies involving differential centrifugation (20) have now been re-examined. While cytoplasmic inclusions were found in the tissue sections in numbers comparable to those reported here, no indication of inclusions could be found in fixed smears of the homogenates or nuclear, large granule, small granule, or supernatant fluid fractions from the same livers.

both Opie (15) and we find in animals fed the azo dyes. It is, on the other hand, also possible that the peripheral recrudescence of parenchymal cells may come from the scattered parenchymal cells trapped among the hyperplastic duct tissue. This possibility must be entertained, although it leaves the mechanism of the disappearance of the bile duct cells unexplained.

Although the majority of the duct cells were eventually replaced by typical parenchymal tissue, scattered islets of hyperplastic ducts persisted. In proportion to the contained fibrous tissue, they were classified as hyperplasia or cholangiofibrosis. Later, when the epithelium assumed a neoplastic appearance, as described by Opie (15), they were regarded as adenomas. Discontinuation of the dye at this time prevented further change in these areas but was not associated with regression. The failure to regress suggests autonomous growth or neoplasia, whereas the inability to progress indicates the benign character of the lesions.

With continued feeding of the dyes, malignant neoplasia arose in most instances from these areas of cholangiofibrosis or adenomas. If 3′-Me-DAB was withdrawn from the diet after 35 days, the adenomas persisted unchanged for as long as 98 days, while other pathological changes, except for a few cysts, disappeared. These observations assume more significance in the light of some recent studies by Clayton and Baumann (2), who showed that rats fed 0.064 per cent of 3′-Me-DAB for only 4 weeks did not develop tumors, whereas liver tumors did develop in animals fed the dye for two 4-week periods separated by as much as a 12-week interval on the basal diet. Thus, some change induced during the early feeding period persisted through the intermediate dye-free period. This persistent predisposition, demonstrated by Clayton and Baumann, may be related to the persistent areas of cholangiofibrosis observed in the present experiment. The subsequent association of areas of cholangiofibrosis with the malignancies produced when the carcinogen was fed continuously strengthens the possibility of such a relationship.

Metastases were not found in this study, probably because the animals were killed as soon as neoplasms were found by laparotomy and since an extensive search was not made for microscopic metastases. The carcinomas induced by 3′-Me-DAB do spread by metastasis in the majority of cases if the animals are kept until they die of the tumors (3, 22).

The histological patterns assumed by the carcinomas have varied from adenocarcinomatous to hepatomatous, with many examples of mixed types. Richardson and Borsos-Nachtnebel (22) have proposed a more elaborate classification of the carcinomas induced in the livers of rats fed 3′-Me-DAB, and we have observed variants resembling those described by these authors. The value of such classification appears arbitrary and limited, because nearly all types, either the "hepatoma" or "cholangioma," seem to arise from foci of cholangiofibrosis or adenomata. The mixed tumors comprised 24 per cent of those studied and were especially interesting in this regard, because, in such tumors, a gradual transition of one type of structure into the other with a merging of both patterns was seen. We regard this as indicative of a common origin and trace their source from the early hyperplastic ducts, through the persistent cholangiofibrosis to their ultimate form. Because of these histogenetic relationships we prefer to consider all these tumors "carcinoma of the liver."

Richardson and Borsos-Nachtnebel (22) have also described sarcomas in the livers of rats fed 3′-Me-DAB. A careful search failed to reveal sarcomas in the livers of rats fed either carcinogen in this laboratory.

SUMMARY

1. Rats were fed a basal diet with or without 0.058 per cent of either 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) or 4′-fluoro-4-dimethylaminoazobenzene (4′-F-DAB), and animals from all groups were selected for histological studies of their livers at frequent intervals until neoplasms were grossly evident. In order to study the reversibility of the early histological changes, another group of animals was transferred to the basal diet for the duration of the experiment after ingesting the ring-methyl dye for 5 weeks.

2. Hyaline droplets and inclusions appeared in the cytoplasm after 7 days of ingestion of either carcinogen, increased in size and number, reached a maximum when the protein-bound dye was present in the largest amounts, and then gradually disappeared as the level of protein-bound dye declined. These inclusions were predominantly peripheral when 3′-Me-DAB and central when 4′-F-DAB was fed, so that by this feature one could determine microscopically which carcinogen had been ingested.

3. Considerable proliferation of the bile ducts occurred, especially when 3′-Me-DAB was fed. The new bile duct cells were the origin of cholangiofibrosis, which persisted for as long as 98 days after the carcinogen was removed from the diet. Most of the bile duct cells disappeared between the sixth and eighth weeks. Evidence is presented which suggests that these bile duct cells were transformed into parenchymal cells.

4. The neoplasms produced by these carcinogens were very similar and resembled those produced by the less active 4-dimethylaminoazobenzene. It appears that most—if not all—of these neoplasms, regardless of their histological patterns, arise from areas of cholangiofibrosis and thus have a common pathogenesis.

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Fig. 1.—Hyaline inclusions from cells about the central vein; liver from rat fed 4′-F-DAB for 28 days. Most of the cells contain the inclusions, which in some places have shrunk from the surrounding cytoplasm, leaving a clear space around the inclusion. Hematoxylin and eosin stain was used for all these sections unless otherwise noted. ×725.

Fig. 2.—Liver lobule from rat fed 3′-Me-DAB for 24 days. There is an increased basophilia of peripheral nuclei and very early bile duct hyperplasia. ×75.

Fig. 3.—Definite bile duct hyperplasia around a liver lobule from a rat fed 3′-Me-DAB for 26 days. ×75.

Fig. 4.—Liver lobule from rat fed 3′-Me-DAB for 28 days. Over two-thirds of the parenchymal cells of this lobule have been replaced by bile duct cells. Note the lack of distortion of the lobular structure. ×75.

Fig. 5.—Portion of a lobule from a rat fed 3′-Me-DAB for 32 days. There is a tendency to duct formation in the area of new bile duct cells. The central vein is just below the lower central portion of the photograph. ×160.

Fig. 6.—Section showing one of the early alterations in the appearance of the new bile duct cells formed in the liver of a rat fed 3′-Me-DAB for 30 days. The large, basophilic ducts are surrounded by newly formed bile duct cells. ×180.

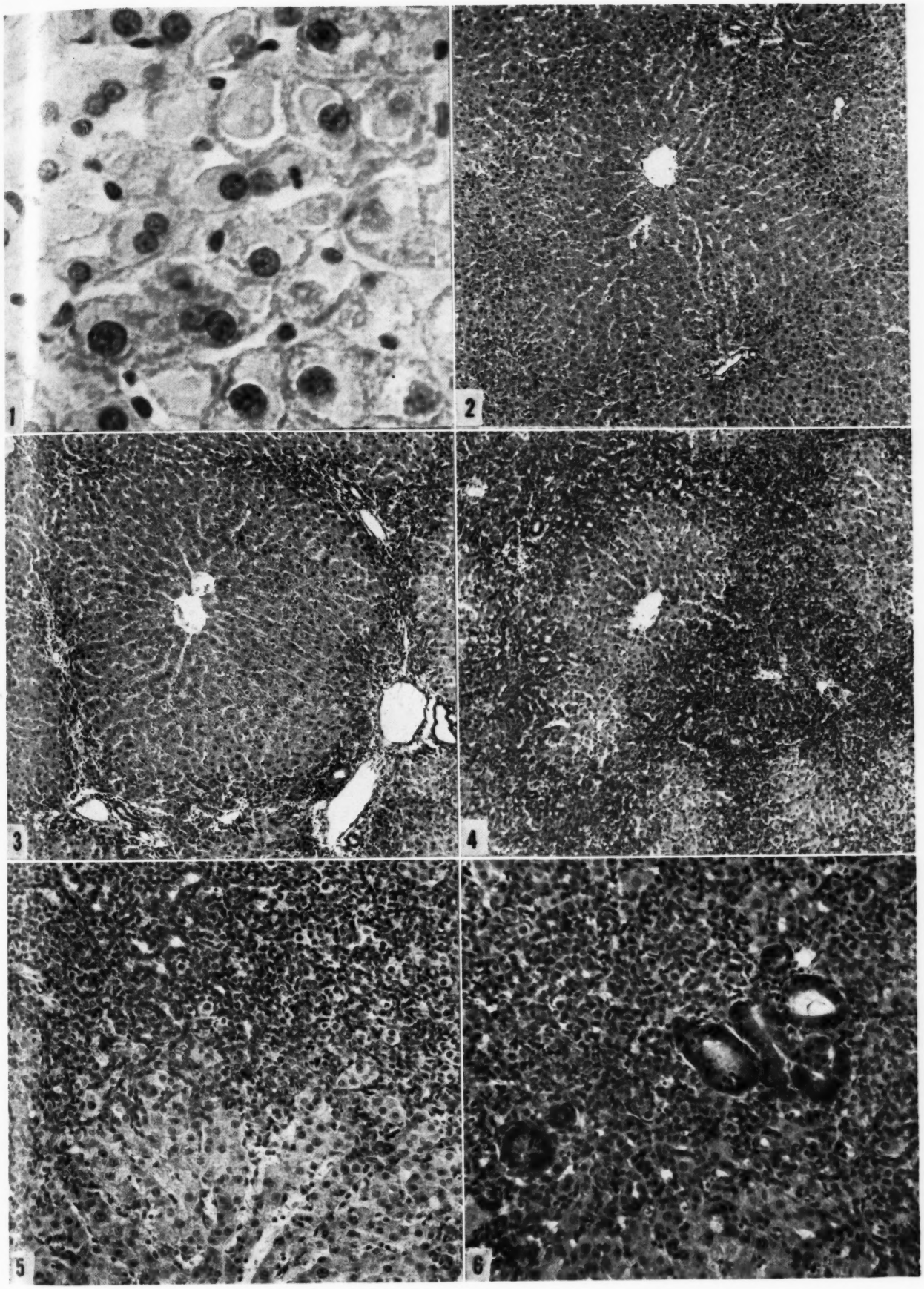
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FIG. 7.—Area of large bile duct cells in dense connective tissue, or "cholangiofibrosis." This animal had been fed 3′-Me-DAB for 63 days. ×160.

FIG. 8.—Section showing the change in the appearance of the bile duct cells during the period when they were increasing in size and assuming the morphological characteristics of liver cells. Three nearly complete lobules and parts of others are shown. In those lobules that are nearly complete, the cells about the central vein are large and eosinophilic, while those in the periphery are more basophilic. The peripheral cells should be compared with those shown in Figure 4. This animal was fed the ring-methyl dye for 35 days and then fed the basal diet for 5 days. As a result, most of the cells are in about the same stage of conversion, so that this section is particularly good for the demonstration of a change that could easily be demonstrated in other animals at this time, although they were fed the carcinogen without interruption. ×75.

FIG. 9.—This section shows a later stage in the apparent conversion of bile duct cells to cells resembling liver cells. The former bile duct cells uppermost in the picture are separated from the original liver cells below by a thin strand of connective tissue. ×160.

FIG. 10.—This section is from the same liver as that shown in Figure 8, but here the tissue has been stained for mitochondria by Mallory's phosphotungstic acid hematoxylin. The numerous mitochondria in the parenchymal cells around the central veins make those cells appear very basophilic, while the bile duct cells are lighter colored. ×75.

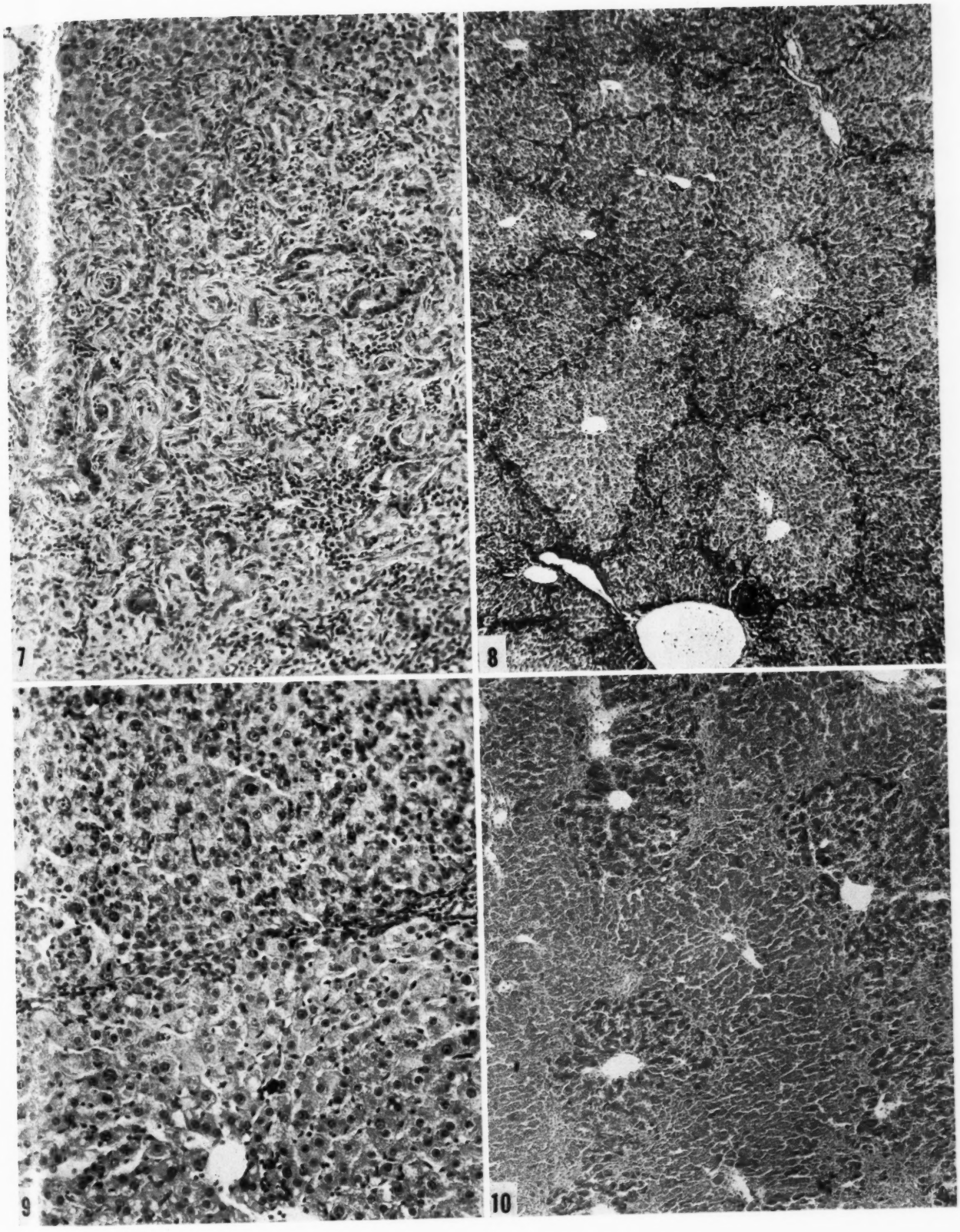
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FIG. 11.—Cells from the periphery of the lobules shown in Figure 10. Note the relative paucity of mitochondria. Stained as for Figure 10. ×2,000.

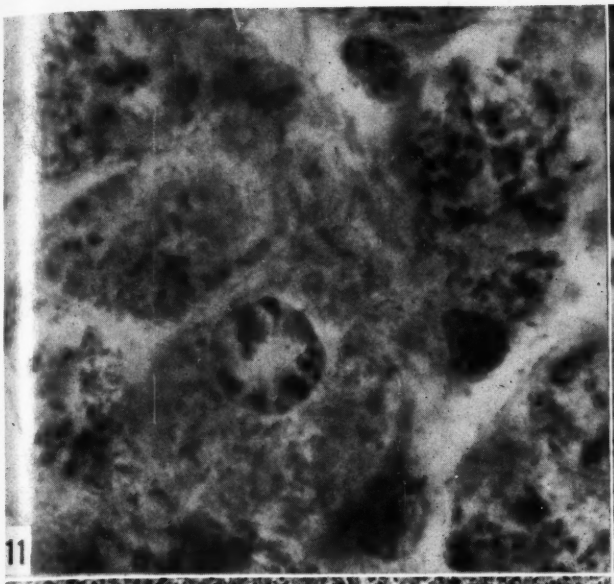
FIG. 12.—Cells from around the central vein showing the relatively large numbers of mitochondria in the parenchymal cells. See Figures 10 and 11 for comparison. Stained as for Figure 10. ×2,000.

FIG. 13.—Section showing the type of necrosis that was found around the central vein when 4′-F-DAB was fed for 32 days. There has been extravasation of blood into the areas formerly occupied by the liver cells. ×75.

FIG. 14.—Somewhat more advanced changes than those shown in Figure 13. Besides the necrosis about the central veins there has been moderate bile duct proliferation. There are small clusters of lymphocytes scattered throughout the field. ×75.

FIG. 15.—A nodule of regenerating parenchymal cells which has appeared after only 30 days of ingestion of the ring-methyl dye. Such nodules are seen after this time when either dye is fed, and contribute to the distortion of the liver architecture. ×75.

FIG. 16.—Liver from rat following 63 days of ingestion of the halogen-containing carcinogen. In the center there is an area of cholangiofibrosis, bile duct cells, and fibrous connective tissue. Above there is a nodule of parenchymal cells with nuclei which vary considerably in size. On the lower left there is a nodule of cells that probably was derived from bile duct cells. The cells on the extreme right are some relatively normal parenchymal cells. ×75.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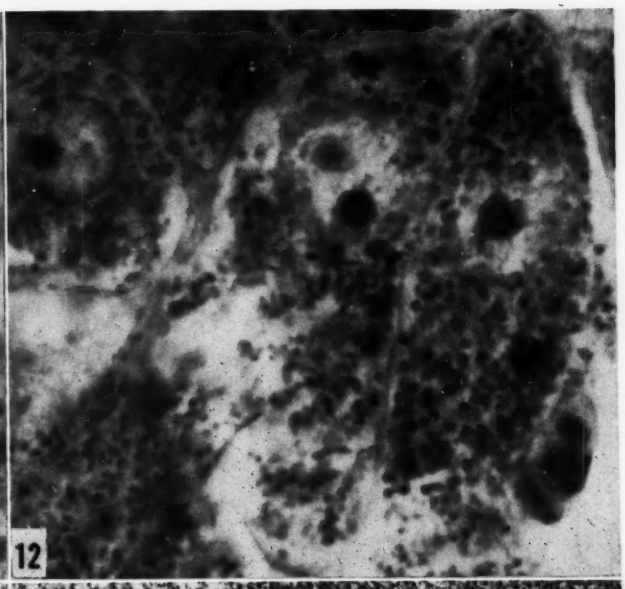
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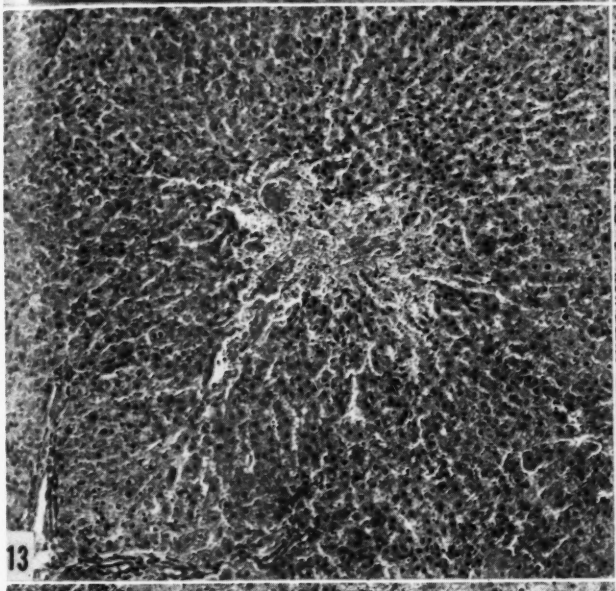
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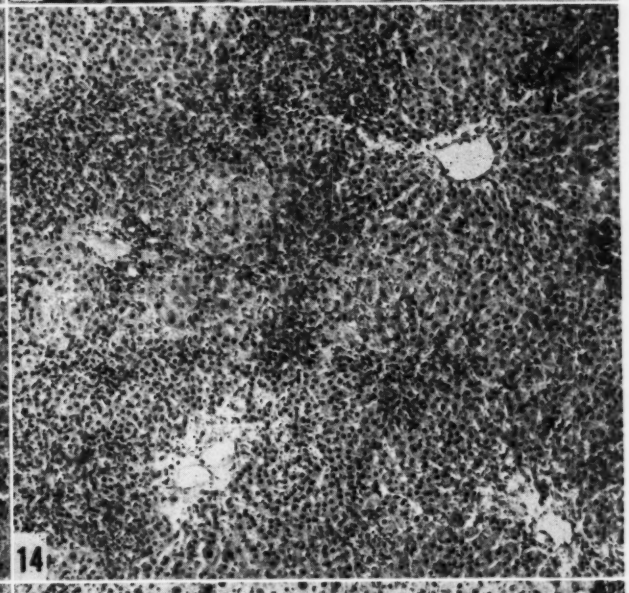
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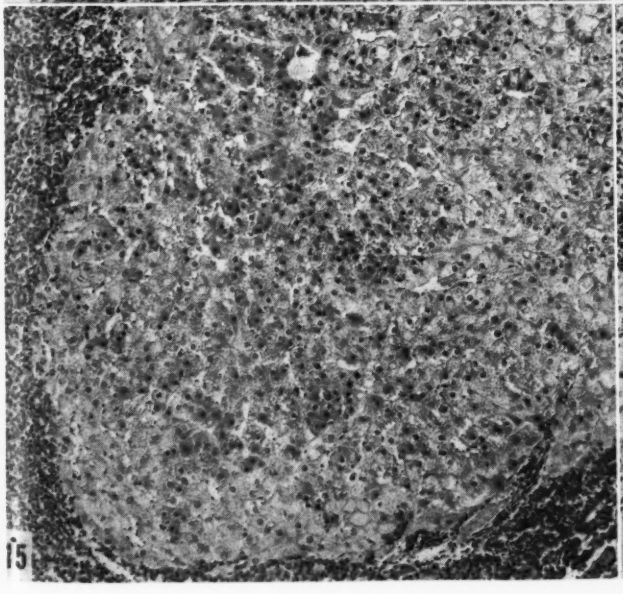
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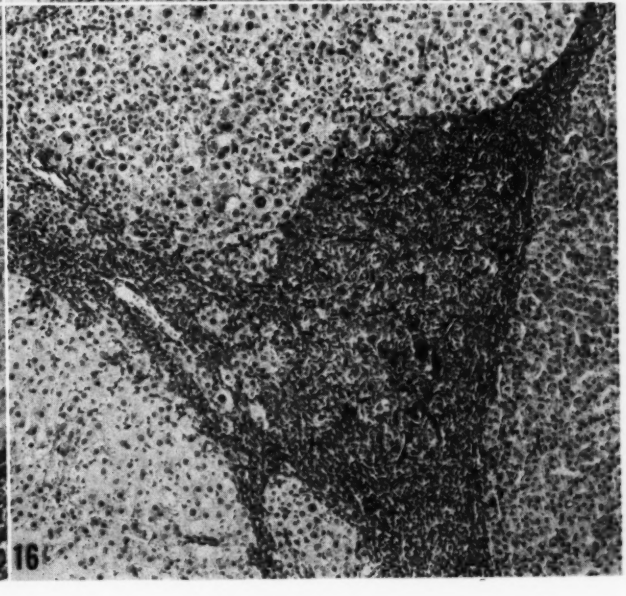
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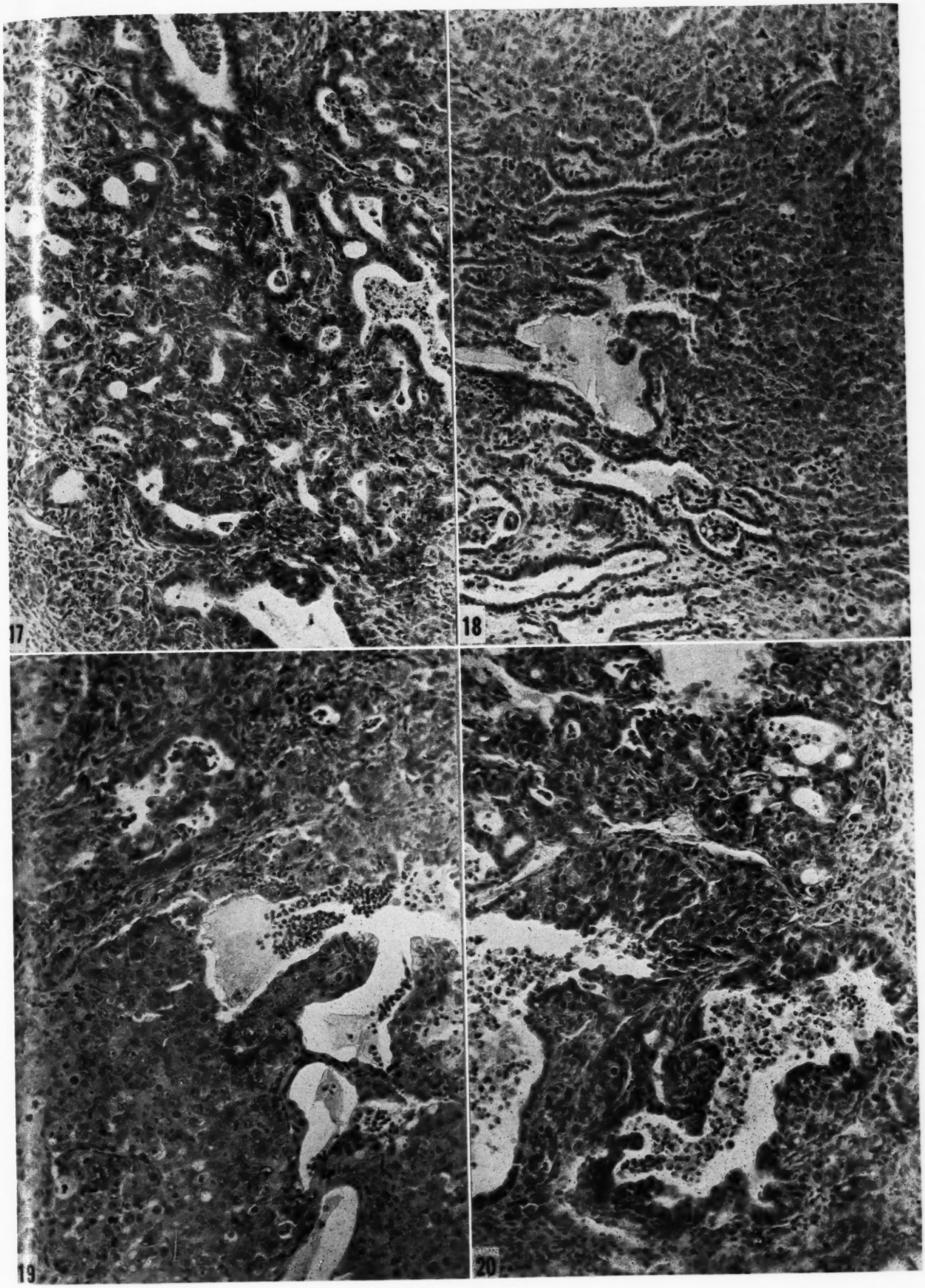
FIG. 17.—Cholangioma induced by feeding 3′-Me-DAB for 133 days. ×160.

FIG. 18.—Mixed tumor in the liver of a rat fed 4′-F-DAB for 119 days. ×160.

FIG. 19.—Hepatoma from the liver of a rat fed 3′-Me-DAB for 119 days. ×160.

FIG. 20.—Cholangioma from liver of rat fed 3′-Me-DAB for 133 days. ×160.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Tumor Incidence and Lethal Mutation Rate in Drosophila Treated with 20-Methylcholanthrene*

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Tumors in Drosophila are in many ways similar to atypical growths in mammals, although they are dissimilar in others (6). A frequent question concerning these tumors is whether carcinogens alter their incidence. This has been tested by treating a tumor stock of Drosophila with 20-methylcholanthrene and is reported at this time. In a previous study (4) it was shown that the lethal mutation rate is unchanged in the Oregon R stock after treatment with this carcinogen. Supplementary data on lethal mutation rate obtained in the present investigation are thought to have additional value, because tumor incidence was tabulated at the same time.

METHODS

The *tu 36a* strain was used in these studies, and, for every culture of flies undergoing treatment, a control culture was tested under laboratory conditions as nearly identical as possible except for treatment with the carcinogen. Only those individuals from groups of siblings with a normal sex ratio were used. Fifty males and 50 females (P_1) from several groups were mated and treated when 3 days of age. In the first experiment the treatment consisted of exposing the flies to an aerosol of 1 per cent methylcholanthrene in sesame oil for 30 seconds every 30 minutes for a period of 24 hours in one group and 48 hours in another. The aerosol was generated by air flowing through a nebulizer at the rate of 6 l. per minute. Individuals in the next generation (P_2) were examined for tumors, and representative females were tested for lethal mutations by the Muller-5 method. Effects of the carcinogen on the germ cells of either or both sexes in the previous generation were detected by using the females from this generation for mating to Muller-5 males. This scheme for detecting tumor incidence and lethal mutation rate simultaneously is given in detail in a previous communication (5).

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In the second experiment, Tween-80[1] was substituted as the solvent for a similar concentration of the carcinogen. It was hoped that this solvent would enhance the effectiveness of the carcinogen. The tumor incidence was determined in the P_2 generation and in the offspring (F_1) of that generation as well. An isogenic, instead of a selected, *tu 36a* stock was used, and the experiment was carried out in an incubator at 25 ± 0.5° C. This isogenic stock was obtained by mating the tumor strain to appropriate stocks with chromosome aberrations to prevent crossing over in the resulting heterozygotes and then backcrossing so that both chromosomes in each pair were derived from a single antecedent chromosome.

TABLE 1

EFFECT OF 20-METHYLCHOLANTHRENE IN SESAME OIL (24-HOUR ADMINISTRATION) ON TUMOR INCIDENCE IN P_2

	Tumors	Population	Per cent tumorous	P
♂♂ Treated	9	221	4.07	0.038
♂♂ Without treatment	6	382	1.57	
♀♀ Treated	15	312	4.81	<0.0001
♀♀ Without treatment	2	482	0.41	
Total treated	24	533	4.50	<0.0001
Total without treatment	8	864	0.93	

RESULTS

Experiment 1.—There were 24 tumorous individuals among 533 flies (4.5 per cent) in the group treated for 24 hours and only 8 among 864 (0.93 per cent) in the control group ($P < 0.0001$) (Table 1). A higher incidence in the treated group was also present when the incidences for males ($P = 0.038$) and females ($P < 0.0001$) were examined separately. A larger group was treated for 48 hours (Table 2), and again the males, females, and total

[1] Polyoxyethylene sorbitan monooleate obtained from the Atlas Powder Co., Wilmington, Del.

number in the treated group included a significantly higher incidence of tumorous flies than in the group without treatment ($P < 0.0001$). There were only 23 tumorous flies from a control population of 1,927, as compared to 126 tumorous individuals from a population of 2,837 in the group treated for 48 hours. It is interesting that the incidence of tumors was very similar for the two periods of treatment, even though the carcinogen was administered twice as long in the second group (Table 1 and 2).

After treatment, the flies were allowed to lay eggs on culture medium in the same bottle. In 3 days they were transferred to a fresh culture. Control groups were treated in the same manner. The data in Tables 1 and 2 for treated and control groups are separated into results for original and transfer cultures in Table 3. There is no significant difference in tumor incidence between the original and transfer cultures except in the females from the treated group. It should be pointed out that the original and transfer cultures were not observed during the same period of time. When both original and transfer cultures of treated groups are compared to those without treatment there is a pronounced difference in tumor incidence.

TABLE 2

EFFECT OF 20-METHYLCHOLANTHRENE IN SESAME OIL (48-HOUR ADMINISTRATION) ON TUMOR INCIDENCE IN P_2

	Tumors	Population	Per cent tumorous	P
♂♂ Treated	64	1,374	4.66	<0.0001
♂♂ Without treatment	9	876	1.03	
♀♀ Treated	62	1,463	4.24	<0.0001
♀♀ Without treatment	14	1,051	1.40	
Total treated	126	2,837	4.44	<0.0001
Total without treatment	23	1,927	1.19	

In contrast to the effect of 20-methylcholanthrene on tumor incidence, there was no significant difference in mutation rate after it was administered for either 24 (Table 4) or 48 hours (Table 5), when compared to control cultures. There were 2 lethals among 760 chromosomes tested after treatment for 24 hours, compared to 2 among 913 chromosomes tested from flies without treatment ($P = 0.96$). For the 48-hour treatment period there were 12 lethals among 2,413 chromosomes tested and 7 lethals among 2,366 chromosomes tested in the control group ($P = 0.26$).

Experiment 2.—In the second experiment, with an isogenic stock with less variation in temperature, the tumor incidence was also higher after treatment with 20-methylcholanthrene in Tween-80. The carcinogen was only administered for 24 hours, because of the results in the previous experiment. In Table 6, there are 30 (3.55 per cent) tumorous flies among 845 in the P_2 generation of the treated groups, as compared to 16 (2.15 per cent) among 744 in the same generation without treatment ($P = 0.099$). The incidence both in males and in females is higher with treatment when the sexes are examined separately. It will be

TABLE 3

TUMOR INCIDENCE IN ORIGINAL AND TRANSFER CULTURES

	Culture	Tumors	Population	Per cent tumorous	P
♂♂ Treated	Original	56	1,165	4.81	0.48
	Transfer	17	430	3.95	
♀♀ Treated	Original	52	1,391	3.74	0.02
	Transfer	25	384	6.51	
Total treated	Original	108	2,556	4.23	0.28
	Transfer	42	814	5.16	
♂♂ Without treatment	Original	6	801	0.75	0.06
	Transfer	9	457	1.97	
♀♀ Without treatment	Original	13	1,185	1.10	0.71
	Transfer	3	348	0.86	
Total without treatment	Original	19	1,986	0.96	0.22
	Transfer	12	805	1.49	

TABLE 4

EFFECT OF 20-METHYLCHOLANTHRENE IN SESAME OIL (24-HOUR ADMINISTRATION) ON LETHAL MUTATION RATE

	Lethals	Chromosomes tested	Per cent lethals	P
Treated	2	760	0.26	0.96
Control	2	913	0.22	

TABLE 5

EFFECT OF 20-METHYLCHOLANTHRENE IN SESAME OIL (48-HOUR ADMINISTRATION) ON LETHAL MUTATION RATE

	Lethals	Chromosomes tested	Per cent lethals	P
Treated	12	2,413	0.50	0.26
Control	7	2,366	0.30	

noted, however, that these differences are not statistically significant.

An increase in tumor incidence with treatment was again encountered in the F_1 generation when the sexes were considered together or separately (Table 7). There was a total of 12 flies (5.04 per cent) with tumors among 238 individuals in the treated group and 5 flies (1.21 per cent) among 414 in control cultures ($P = 0.003$).

Lethal mutation rate was unaffected by the treatment. It may be seen in Table 8 that there were no lethals in the 1,091 chromosomes tested following treatment and 1 lethal in 1,281 chromosomes tested in the flies without treatment ($P = 0.36$).

TABLE 6

EFFECT OF 20-METHYLCHOLANTHRENE IN TWEEN-80 (24-HOUR ADMINISTRATION) ON TUMOR INCIDENCE IN P_2

	Tumors	Population	Per cent tumorous	P
♂♂ Treated	16	404	3.96	0.32
♂♂ Without treatment	9	338	2.66	
♀♀ Treated	14	441	3.17	0.18
♀♀ Without treatment	7	406	1.72	
Total treated	30	845	3.55	0.10
Total without treatment	16	744	2.15	

TABLE 7

EFFECT OF 20-METHYLCHOLANTHRENE IN TWEEN-80 (24-HOUR ADMINISTRATION) ON TUMOR INCIDENCE IN F_1

	Tumors	Population	Per cent tumorous	P
♂♂ Treated	4	112	3.57	0.042
♂♂ Without treatment	1	192	0.52	
♀♀ Treated	8	126	6.35	0.025
♀♀ Without treatment	4	222	1.80	
Total treated	12	238	5.04	0.003
Total without treatment	5	414	1.21	

TABLE 8

EFFECT OF 20-METHYLCHOLANTHRENE IN TWEEN-80 (24-HOUR ADMINISTRATION) ON LETHAL MUTATION RATE

	Lethals	Chromosomes tested	Per cent lethals	P
Total treated		1,091		0.36
Total control	1	1,281	0.08	

The tumors varied widely in size. This was computed as length × width, measured with a calibrated, ocular micrometer (Table 9). No constant difference could be found between the size of tumors appearing in the treated and the control groups or in flies of different sizes. For example, with 20-methylcholanthrene treatment, the average size of tumors in males was smaller than in the control groups, but the reverse was true for females.

TABLE 9

SIZE OF TUMORS

	No. tumors	Av. size of tumors (sq. mm.)	Av. length of body (mm.)
♂♂ Treated	20	0.0064	2.27
♂♂ Without treatment	10	0.0103	2.34
♀♀ Treated	22	0.0120	2.72
♀♀ Without treatment	11	0.0056	2.78
Total treated	42	0.0093	2.50
Total without treatment	21	0.0075	2.57

DISCUSSION

The evidence in Tables 1, 2, 6, and 7 indicates in a uniform manner that the incidence of tumors in this strain is increased when 20-methylcholanthrene is administered as an aerosol. Although the number of tumors appearing after treatment is not dramatic, the increase seems nonetheless real. Personal observations and communications from others indicate that tumors appear sporadically in natural populations of Drosophila. There is, therefore, a certain incidence of tumors even in the wild state, and conclusions based on the effect of a carcinogen on incidence in a tumor stock should be equally applicable to wild populations.

Evidently, in the concentration used no additional effect is obtained after a certain dosage is reached, judging from the similar incidence of tumors following both 24- and 48-hour administration of the carcinogen (Tables 1 and 2). This is similar to the finding in mammals that optimal effects do not necessarily parallel the magnitude of the dose. This may also explain why offspring raised in food on which the carcinogen was sprayed during treatment of the parent did not necessarily have a higher incidence of tumors than individuals in transfer cultures (Table 3).

One of the most convincing phases of this study has been that *the incidence of tumors in each individual, treated culture has been greater than that in the parallel, control culture in every instance.* The significance of the findings as a whole is substantiated by the χ^2 test, except in the data for the P_2 generation in Experiment 2, which showed an increased incidence with treatment but which was

not statistically significant. Although a possible delayed effect of the carcinogen appearing in the F_1 is suggested by Table 7, the small numbers involved do not entirely justify such a conclusion. It is interesting that this chemical is tumorigenic in Drosophila as well as in certain other animals.

The nature of the pigmentation is superficially not different in tumors arising either with or without treatment. Sections were not prepared, since observations were made in adults, some of which were used in ascertaining lethal mutation rate. In addition to the data already mentioned in Experiment 2, other evidence that the size of the tumor is not related to treatment comes from Experiment 1. In 4 cultures the average size was greater in those tumors appearing after treatment, and in 5 it was smaller. With 24-hour treatment the average size of 24 tumors was 0.0056 sq. mm. Following 48-hour treatment the average tumor measurement was 0.02 sq. mm. (39 tumors) and 0.012 sq. mm. in tumors from 9 individuals without antecedent treatment. A second group treated 48 hours at a different time had a mean size of 0.0056 sq. mm. (93 tumors), as compared to 0.011 sq. mm. (14 tumors) in the control group.

Early positive results obtained by Sacharov (8) and Demerec in testing the effect of carcinogens on mutation rate in Drosophila have been construed by some workers as additional evidence for the somatic mutation theory of tumorigenesis. Auerbach (1) and Bhattacharya (3) were unable to show a similar effect. Later, Demerec and coworkers (7) repeated their work, and honestly and commendably reported that "the variability from experiment to experiment became alarming and only occasionally was it possible to obtain confirmation of previous experiments. The fourth period, encompassing all the past year, has been characterized by uniformly negative results, except in those experiments using nitrogen mustard, methyl-bis(β-chloroethyl)amine hydrochloride." Methylcholanthrene is among the carcinogens included in the eighteen chemicals yielding negative results when very large numbers of spermatozoa (130,649) were tested by these investigators.

The results obtained in this study also fail to demonstrate a mutagenic action of 20-methylcholanthrene in two separate experiments. In Table 5 the mutation rate is not significantly different between treated and control groups, and it is interesting that the twelve lethals following treatment have six origins and the seven lethals in the control group also have six origins. It seems unlikely that the unchanged mutation rate is the result of failure of the chemical to reach the cell in an active form, since it is effective in increasing tumor incidence. For a similar reason there is apparently no species barrier to the action of the carcinogen in Drosophila.

Combining the evidence from these and previous studies (4), the lethal mutation rate is 0.17 per cent (20 mutations among 11,648 chromosomes tested) after treatment with 20-methylcholanthrene, and 0.18 per cent (11 among 5,971 chromosomes tested) in control cultures. In contrast to this, the tumor incidence is 4.31 per cent (192 among 4,453 individuals) following treatment, and only 1.32 per cent (52 among 3,949 flies) without contact with the carcinogen. These results are compatible with those of Auerbach (1), Bhattacharya (3) and Demerec (7) concerning the lack of mutagenic effect of this carcinogen in Drosophila.

Barratt and Tatum (2) report that methylcholanthrene, 9,10-dimethyl-1,2-benzanthracene, and 1,2,5,6-dibenzanthracene increase mutation frequency in *Nuerospora crassa* approximately fourfold over controls but point out that they are much less active in this regard than are nitrogen mustard and irradiation. No satisfactory explanation can be offered for the discrepancy between the results with Neurospora and Drosophila, particularly since a tumorigenic effect is demonstrable in the latter without any evidence of a simultaneous mutagenic action. Dr. E. L. Tatum suggested that Tween-80 be used as the solvent for the carcinogen, since it has been used in the experiments with Neurospora. Employment of this solvent in Experiment 2 failed to demonstrate an increased mutation rate in Drosophila, however.

If the somatic mutation theory is valid, it is necessary first to show that carcinogens are mutagens and second that this mutagenic property is directly responsible for tumorigenesis. In this study it is apparent that neither point has been demonstrated, although tumors have increased in numbers with administration of the carcinogen during the same interval for testing the mutation rate. Appropriate restraint in drawing conclusions is always in order, particularly if one is conversant with past experience in the field of oncology. It does appear, however, that recent evidence does not indicate that 20-methylcholanthrene is mutagenic in Drosophila, or that its tumorigenic effect in these experiments is the result of somatic mutation. The information that a carcinogen may increase tumor incidence in *Drosophila* increases the similarity between these tumors and those in other species.

CONCLUSIONS

1. Tumor incidence was increased in the *tu 36a* strain of Drosophila after treatment with 20-

methylcholanthrene administered as an aerosol.

2. The lethal mutation rate was unchanged by treatment, even though it was determined at the same time that the tumorigenic effect of the carcinogen was demonstrated.

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The Comparative Effects of Various Steroids on Lymphoid Tissue of the Rat*

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Many investigators have shown that the response of lymphoid tissue to adrenocorticotropic hormone (ACTH) is a general effect. The rat (17), the dog (12), the mouse (3), the rabbit and the human (4) all show a characteristic response to the administration of ACTH. When given to adrenalectomized animals, ACTH has no effect on lymphoid tissue, but cortical steroids have. Adrenal cortical extracts, corticosterone, and cortisone have all been reported to produce lymphoid involution when given to adrenalectomized mice (4). Moreover, Dontigny (2) reported that large doses of desoxycorticosterone acetate produce an atrophy of the thymus but not of other lymphoid tissue in adrenalectomized rats. The sex steroids are also capable of producing thymic involution even in the adrenalectomized animal (16).

In the course of our studies, we have investigated the effects of repeated injections of various steroids on the normal adult male rat. This publication concerns the comparative effect of these steroids on adrenal, thymus, and lymph node weights.

MATERIALS AND METHODS

A total of 360 male rats of the Sprague-Dawley strain with a body weight which varied between 250 and 300 gm. was used in these studies. Each group consisted of ten normal, healthy animals of uniform weight.

Injections were made subcutaneously 3 times a day approximately every 8 hours, except for compounds dissolved in oil which were injected twice a day—early morning and late afternoon. All compounds were given for 10 days. Testosterone propionate, progesterone, estrone, estradiol, and estriol were given in sesame oil. The adrenal extract was aqueous. ACTH was dissolved in saline before injection. All other compounds were suspended in saline and given as suspensions.

Since one of our interests in the steroids was their effect on thyroid function, animals were maintained throughout the study on double-distilled water and a low iodine diet (10, 11). No evidence exists that this diet in any way influences the adrenal or lymphoid systems. It is quite probable, however, that some dietary factors may do so (16).

The animals in Series I–V were maintained on this diet for 10 days before treatment was started and were allowed to feed until the time of killing. In Series VI the rats were on a diet high in carbohydrates[1] for 5 days before the injections were started. Food was withdrawn from these animals 24 hours before autopsy.

Twenty-four hours before autopsy all animals were given 1 μc. of carrier-free I^{131} intraperitoneally for thyroid studies. At the time of autopsy (which was done approximately 24 hours after the last steroid injection), the rats were given a small dose of pento-barbital sodium and were exsanguinated from the dorsal aorta. Organs were removed, dissected free of fat and connective tissue, and weighed on a Roller-Smith balance.

EXPERIMENTAL RESULTS

The compounds we have used, the daily dose administered, the body, adrenal, thymus, and mesenteric lymph node weights are given in Table 1. Chart 1 shows the lymph node data comparing the experimental to the control values. This was done to make possible a comparison of experimental groups done at different times. For this we

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[1] Our low iodine diet was dry, and exact estimates of daily intake were difficult to determine. For this series of animals, we changed to a semi-liquid diet used by Ingle (7). The only modification was that we used 5 gm. of cod liver oil and added 200 cc. more of water. Steroid treatment did not significantly influence food consumption in these animals.

arbitrarily considered the control value for each experimental group as 100. The experimental groups were then plotted as a percentage increase or decrease with respect to their controls. These results represent the average for each group of animals.

The influence of these compounds on thyroid function as measured by I^{131} collection has been presented in earlier publications.[2] The following information has been obtained regarding the effect of ACTH and these steroids on the lymphoid system.

[2] Biochemical studies done on blood and other tissues of some of these animals are being reported in a separate publication (1).

Effect on the Weight of the Thymus

Compounds which increased thymic weight.—Progesterone was the only compound which significantly increased the size of the thymus. The results suggest that 21-acetoxypregnenolone (15 mg.) and dihydro E (17α-hydroxy-21-acetoxypregnane-3,11,20-trione) increased thymus weight, but the differences are not statistically significant.

TABLE 1

COMPARATIVE EFFECT OF COMPOUNDS STUDIED

Compound	No. animals surviving	mg/day/rat	Body wt. (gm.)	Adrenal wt. (mg.)	Thymus wt. (mg.)	Lymph node wt. (mg.)
Series I:						
17 hydroxy-20, 21-diacetoxy-pregnen-3-one	10	5.0	266	37.9	439	21.6*
Adrenal extract	10	2.1 (cc.)	261	35.9	383	25.9
Compound S	9	5.0	269	32.4	427	31.2
ACTH	10	5.0 (std.)	227†	76.9†	115†	15.2†
Controls	8		269	33.9	449	27.2
Series II:						
Cortisone	10	2.5	238†	24.4†	112†	6.8†
Testosterone propionate	10	5.0	283	38.2	183†	17.0
Progesterone	9	5.0	293	39.5	486†	16.9
Estrone	8	50 gamma	260	46.6†	341†	21.8
Controls	10		278	38.1	412	17.9
Series III:						
Compound L	10	15.0	284†	39.9	357	30.9†
21-Acetoxypregnenolone	10	15.0	290*	39.2	386	27.9†
Pregnenolone	10	15.0	283*	37.1	268	17.5
Cortisone	11	5.0	230†	20.4†	26.2†	5.1†
Estradiol benzoate	9	1.0	256	66.6†	174†	19.1*
Controls	9		267	40.6	334	14.0
Series IV:						
Testosterone	9	5.0	316	39.4	85*	16.8*
Estradiol benzoate	10	50 gamma	282†	45.0†	187†	14.5
Estriol	10	1.0	275†	66.9†	147†	20.2†
Desoxycorticosterone	9	15.0	336	25.2†	210†	16.6*
Diethylstilbestrol	8	1.0	285†	58.5†	193†	22.2†
Controls	10		321	37.0	338	12.3
Series V:						
Cortisone	10	10.0	238†	21.9†	166†	3.0†
Dihydro E	10	30.0	292	40.2	509	11.4
Compound L	10	30.0	289	47.4†	356*	13.2
21-Acetoxypregnenolone	10	30.0	309*	38.9	482	13.6
Pregnenolone	10	30.0	299	38.6	389	12.8
Compound A	10	20.0	246†	19.6†	111†	2.6†
Controls	10		292	38.9	474	13.0
Series VI:						
Cortisone	8	10.0	208†	25.7†	41.1†	9.8†
Dihydro E	9	30.0	277	48.5*	229	41.5
21-Acetoxypregnenolone	8	30.0	270	49.9	168	67.2*
Compound A	8	20.0	224*	23.8†	49.3†	11.9†
Compound L	10	15.0	251	58.5	173	77.3†
Compound L	7	30.0	247	75.4*	122*	86.0*
Controls	7		269	53.0	203	44.4

* P = <0.05, according to the method of Fisher (5).
† P = <0.01, according to the method of Fisher (5).

Compounds which depressed thymus weight.—The greatest loss of thymus tissue followed the administration of cortisone. The effects seen with ACTH (equivalent to 5 mg. of LAIA) and Kendall's Compound A (11-dehydrocorticosterone acetate) were, however, nearly as marked. Testosterone, likewise, effectively decreased thymus weight. As might be expected from the studies done by others, female sex hormones of the estrogenic type produced thymus involution.

Pregnenolone, desoxycorticosterone and Compound S (17α-hydroxy-11,21-acetoxydesoxycorticosterone) were also found to decrease thymus weight. Compound L (3β-acetoxy-17α-hydroxyallopregnan-20-one) in three of four groups acted in the same manner as cortisone but not to the same degree.

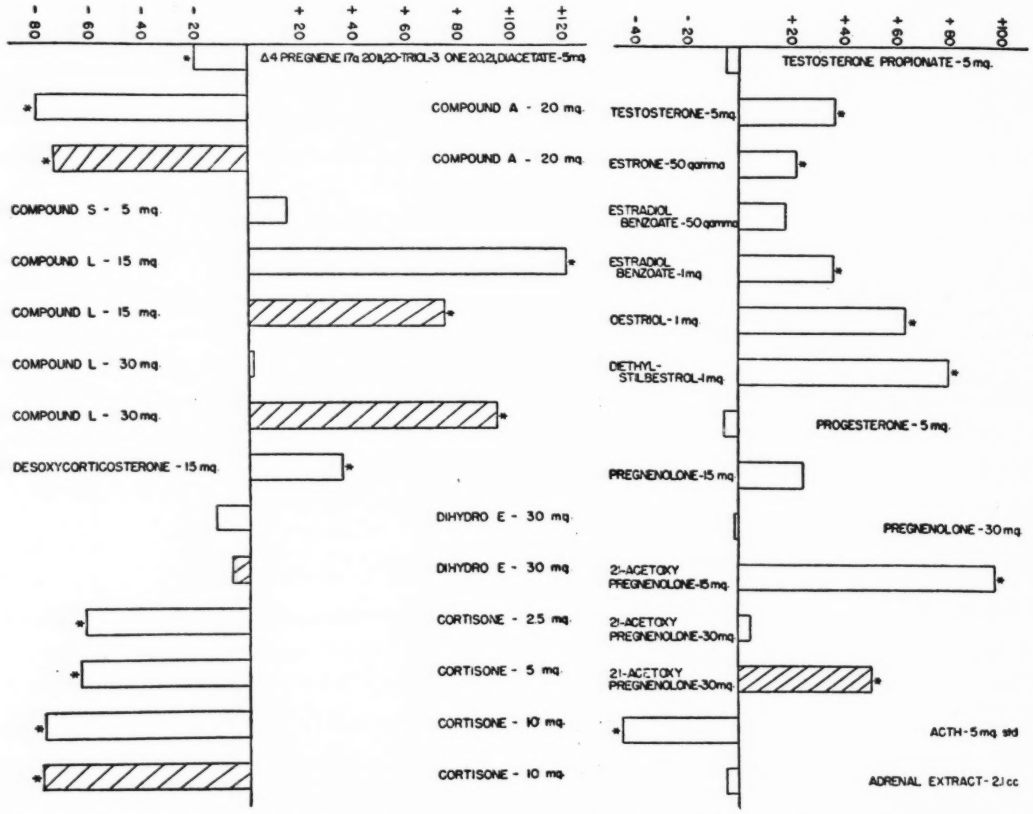

Chart 1 shows a comparative plot of the mesenteric lymph node weights (see Table 1). We have arbitrarily considered the control value for each experimental group as 100 and plotted the experimental groups as a percentage increase or decrease with respect to their controls. The center line indicates the control value. Blocks marked with diagonal lines received the high carbohydrate diet. All compounds were given for 10 days. Open blocks received a low iodine diet.

* Indicates P value of less than 0.05 as shown in Table 1.

The Response of Mesenteric Lymph Nodes

Compounds which increased lymph node size.—Chart 1 shows the effects of these compounds on lymph node weight. It is apparent that both the natural and synthetic estrogenic substances increase lymph node size when administered under our experimental conditions. The greatest response followed the administration of diethylstilbestrol, although estriol was nearly as effective.

Testosterone produced a significant increase in lymph node size, whereas testosterone propionate induced a slight but not significant decrease in size.

A dose of 15 mg. daily of pregnenolone increased lymph node weight, while 30 mg. was ineffective in changing the nodes. Lymph node size was increased in two out of three groups of animals treated with 21-acetoxypregnenolone, the greater increase occurring in the animals receiving 15 mg. daily.

Desoxycorticosterone in the dose tested produced a small but significant increase in lymph node weight. Compound S gave an increase which was not, however, significant.

The most striking enlargement of lymph nodes has been observed following administration of Compound L. In three out of four groups of animals recorded in Chart 1, it can be seen that the

nodes increased from 80 per cent to 120 per cent over the control values.

Compounds which decreased lymph node tissue.—ACTH, cortisone, and Compound A all produced a striking loss of lymph node tissue. Examination of Table 1 shows that both cortisone and Compound A produced almost complete destruction of lymph nodes.

17*α*-Hydroxy-21-acetoxypregnane-3,11,20-trione (dihydro E), although producing an apparent decrease in lymph node weight does so only slightly and not to a significant degree.

DISCUSSION

Many investigators have shown that ACTH and the estrogenic hormones produce marked hypertrophy of the adrenals (14, 16). In contrast to the effect of these substances, cortisone and Compound A produce adrenal involution. Dihydro E (17*α*-hydroxy-21-acetoxypregnane-3,11,20-trione), in amounts 12 times greater than the smallest dose of cortisone we used, had little effect on adrenal weight. Our studies with Compound L indicate that this steroid, in high doses, will produce adrenal hypertrophy. Estriol, which has little biological activity as an estrogenic substance, produced essentially the same degree of adrenal hypertrophy as the same amount of estradiol.

Nearly all the steroids that were used produced thymic involution. The most effective steroids in this respect were cortisone, Compound A, and testosterone. Interestingly enough, 2.5 mg. of cortisone was as effective as 10 mg. of cortisone or 20 mg. of Compound A. Here again, as in the case of the adrenals, estriol was observed to be as effective as the same amount of estradiol.

ACTH, cortisone, and Compound A produced intense lymph node involution. A dose of 17*α*-hydroxy-21-acetoxypregnane-3,11,20-trione, even larger, induced little change in the size of the lymph nodes. It is interesting that the direction of lymph node response was found to be the same with testosterone, the estrogens, and Compound L.

In general, we have observed only slight discrepancies in the results obtained in various groups of animals. The same compound tested on different groups of animals did not always produce the same degree of change, but in most cases the differences were slight. There are, however, a few exceptions to this statement. They all concern the effects on lymph nodes. In one series of animals, the response obtained with 30 mg. of 21-acetoxypregnenolone was not so great as with 15 mg. of this steroid. The same was noted with 30 mg. of pregnenolone, in comparison to the effect obtained with 15 mg. Also, in one experiment, animals which were given 30 mg. of Compound L showed no essential change in lymph nodes. In all other animals treated and reported in this paper, Compound L was found to increase the size of the mesenteric nodes. The possible reasons for these variations are being investigated.

It has been shown by others (8) that many of the adrenal steroids produce atrophy of the rat thymus. The effects of the 11-oxygenated steroids on the lymph nodes are similar to those produced in the thymus but not to the same degree. However, we have shown that several compounds will decrease the weight of the thymus but increase the size of the lymph nodes. Throughout this study, we have examined mesenteric nodes located at the bifurcation of the dorsal aorta. Whether all nodes respond in the same way is being investigated.

Cortisone and 11-dehydrocorticosterone have been shown to produce degeneration of lymphocytes in tissue cultures of lymph nodes (6). Schrek (15) found that adrenal cortical extracts, corticosterone, and 17-hydroxycorticosterone *in vitro* had a cytotoxic action on thymus cells of the rabbit. Therefore, one might conclude that these compounds act directly on lymphoid tissue in the intact animal. This would, however, fail to explain why some of the compounds we have used produced thymus regression and lymph node hypertrophy. This latter response becomes more complicated when one considers the study of Savard and Homburger (13), who reported that transplantation of Sarcoma 180 to mice produces atrophy of the thymus with concurrent lymph node hyperplasia. These changes were not completely prevented by hypophysectomy of the animals. Another study along similar lines was done by Marder (9), who reported thymus and lymph node hypertrophy in adrenalectomized mice treated with thyroxine. These studies might suggest that some of the effects we have obtained on lymph nodes may not be specific for the various steroids. This possibility and the mechanism of action on the lymph nodes are being further investigated.

SUMMARY

The effects of ACTH and various steroids on the adrenal gland, thymus, and lymph node weights have been studied. ACTH and the estrogenic steroids have been observed to produce marked adrenal hypertrophy. 11-Dehydrocorticosterone and cortisone produced extreme adrenal involution.

Of the steroids used, progesterone was the only one which produced a significant increase in the size of the thymus. All other compounds produced decreases of various degrees. The most effective

steroids in this respect were cortisone, 11-dehydrocorticosterone, and testosterone.

ACTH, cortisone, and 11-dehydrocorticosterone produced intense lymph node involution. Testosterone, the estrogens, and Reichstein's Compound L all produced increased size of the lymph nodes.

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Protective Action of Stock Diets against the Cancer-Inducing Action of 2-Acetylaminofluorene in Rats*

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INTRODUCTION

Investigations in this laboratory on the carcinogenic action of 2-acetylaminofluorene have revealed that the type of diet fed with the carcinogen can influence the incidence and types of tumors induced in rats. Female rats of the Alabama Experiment Station (AES) strain uniformly developed mammary tumors in about 4 months when they were fed this carcinogen on a semi-purified diet (7). Varying the riboflavin content of the same diet failed to influence mammary tumor induction, but supplements of teropterin or a synthetic detergent slightly enhanced mammary tumor formation (8). High levels of dietary protein inhibited mammary tumor formation (6).

When rats were fed semi-purified or purified diets that were low in fat, the induction of mammary tumors was almost completely inhibited, and tumors in the orbital cavity appeared rather frequently (30 per cent incidence) (9). In the same study, it was also noted that caloric intake has an important bearing on the induction of mammary tumors. Female rats that would be expected to develop mammary tumors in 90–100 per cent of the cases under a condition of adequate food intake developed this type of tumor in only about 50 per cent of cases under a condition of low food intake. These results thus suggest that the type of diet and the amount of diet fed to rats can have a marked influence on 2-acetylaminofluorene carcinogenesis.

The present experiments were conducted to determine the carcinogenicity of 2-acetylaminofluorene when it was fed in diets of natural foodstuffs—i.e., adequate stock diets or modifications of such diets.

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PROCEDURE

Weanling female rats of the AES strain, 35–65 gm. in body weight and 19–23 days of age, served as experimental animals. In addition, one group of twelve Sprague-Dawley weanling female rats (40–50 gm. body weight) was included in the study to compare its tumor susceptibility to that of the AES strain. The animals were kept in individual screen-bottomed cages; they were supplied with ad libitum quantities of feed and water daily, and a record of food consumption was kept during the first 16 weeks of the experiment. The diets were prepared at about 20-day intervals and were stored in a refrigerator.

The animals were examined weekly for surface tumors from the third month until the end of the experiment. At termination, all tumors and grossly abnormal tissues were carried through routine procedures for histologic study. The complete histological description of these tumors will be reported in another publication.

The composition of the diets is given in Table 1. The diets used fall broadly into one of three classes: (*a*) semi-purified, (*b*) modified stock, and (*c*) stock diet. Diet C-159 is identical with the diet that has been used in this laboratory for a number of years as the stock diet for the breeding colony of the AES strain of rats. Diets C-121, C-122, and C-160 are similar to the colony stock diet, except that rice was used as a replacement for wheat and molasses (C-122), or the molasses was replaced either with the wheat (C-121) or with sucrose (C-160).

The diets designated as modified stock diets were similar to the stock diet, except that either the wheat and molasses (diet C-119) or the meat and bone scrap, skim milk powder, alfalfa leaf meal, and molasses (diet C-120) were replaced with sucrose or degerminated corn grits. Water-washed casein, at the 9 per cent dietary level, was used in place of commercial casein.

Diet C-5 has been used extensively in studies with 2-acetylaminofluorene in this laboratory and is representative of semi-purified diets that will promote a high incidence of mammary tumors.

RESULTS

The results (Table 2) with the semi-purified diet C-5 are essentially similar to the results reported earlier (9). Rats on this diet attained an average body weight of 173 gm. in 16 weeks. Twenty-one of the 23 rats developed mammary tumors in an average period of 17 weeks (90 per cent incidence). The incidence of ear duct and liver tumor was between 50 and 60 per cent, which is the range reported earlier.

Rats receiving diets C-161 and C-162 likewise developed a high incidence of mammary, ear duct, and liver tumors. These diets are relatively low

TABLE 1

PERCENTAGE COMPOSITION OF THE DIETS*

	Type of diets								
	Semi-purified diets			Modified stock diets		Stock diets			
Ingredient	C-5	C-161	C-162	C-119	C-120	C-121	C-122	C-159	C-160
Water-extracted casein	9.0			9.0	9.0	12.0	12.0		
Commercial casein		18.0	18.0					12.0	12.0
Degerminated corn grits	20.0			20.0					
Whole wheat					61.5	61.5		56.5	56.5
White rice							61.5		
Meat and bone scrap				10.0		10.0	10.0	10.0	10.0
Skim milk powder				8.0		8.0	8.0	8.0	8.0
Alfalfa leaf meal				2.0		2.0	2.0	2.0	2.0
Molasses			5.0					5.0	
Iodized salt						0.5	0.5	0.5	0.5
Sucrose	51.0	71.0	66.0	31.0	9.5				5.0
Salt mixture 5†	4.0	4.0	4.0	4.0	4.0				
Cod liver oil‡	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lard	15.0	6.0	6.0	15.0	15.0	5.0	5.0	5.0	5.0
Calculated composition:									
Protein, per cent	10.6	18.0	18.0	18.9	15.2	26.5	26.5	26.0	26.0
Fat, per cent	16.2	7.0	7.0	17.3	17.8	8.9	8.9	8.9	8.9

* 2-Acetylaminofluorene was added to all diets at a level of 300 mg/kg. With the exception of diets C-159 and C-160, all diets were fortified with the following B-vitamins (mg/kg): thiamine, 2; pyridoxine, 2; riboflavin, 4; calcium pantothenate, 10; niacin, 20; i-inositol, 200; and choline chloride, 2,000. Diet C-5 also contained 0.3 per cent of added L-cystine.

† J. Nutrition, 33:155–68, 1947.

‡ With the exception of diet C-159, the cod liver oil was fortified with equal parts of α-tocopherol and α-tocopherol acetate to supply 25 mg. of each per kilogram of diet.

TABLE 2

INFLUENCE OF DIET ON TUMOR INDUCTION TIME AND INCIDENCE IN RATS FED 2-ACETYLAMINOFLUORENE

Type of diet	No. of rats	Av. body weight (16-wk.) (gm.)	Av. daily food intake (gm.)	Av. survival (wks.)	No. of animals with tumors*			
					Mammary	Ear duct	Liver	None
Semi-purified diet:								
C-5	23	173	6.6	27	21 (17)	12 (21)	14	0
C-5†	12	140	5.2	26	11 (17)	6 (22)	5	0
C-161	6	222	8.5	26	5 (16)	3 (21)	4	0
C-162	6	229	9.3	27	6 (16)	6 (23)	4	0
Total	47				43	27	27	0
Modified stock diet:								
C-119	6	176	6.6	34‡	4 (20)	3 (26)	1	0
C-120	6	185	6.7	34‡	6 (22)	3 (25)	1	0
Total	12				10	6	2	0
Stock diet:								
C-121	6	177	7.1	34‡	2 (28)	0	2	3
C-122	6	203	8.3	34‡	2 (29)	1 (32)	0	3
C-159	6	221	9.7	42	3 (31)	6 (37)	2	1
C-160	6	209	8.4	41	1 (29)	4 (35)	3	0
Total	24				8	11	7	7

* The numbers in parentheses are the average tumor induction periods in weeks.

† Sprague-Dawley rats (40–50-gm. initial body weight).

‡ These animals were sacrificed at the end of 34 weeks to determine the incidence of liver tumors.

in fat as compared to diet C-5, and the results thus emphasize that a high fat intake is not an essential factor in the consistent production of mammary and other type tumors by feeding 2-acetylaminofluorene.

Rats of the Sprague-Dawley strain receiving diet C-5 developed a high incidence (92 per cent) of mammary tumors on an unusually low food intake and, consequently, a low carcinogen intake. As reported earlier (9), rats of the AES strain, which are limited to the same extent in growth and food intake, develop mammary tumors in only about 50 per cent of cases.

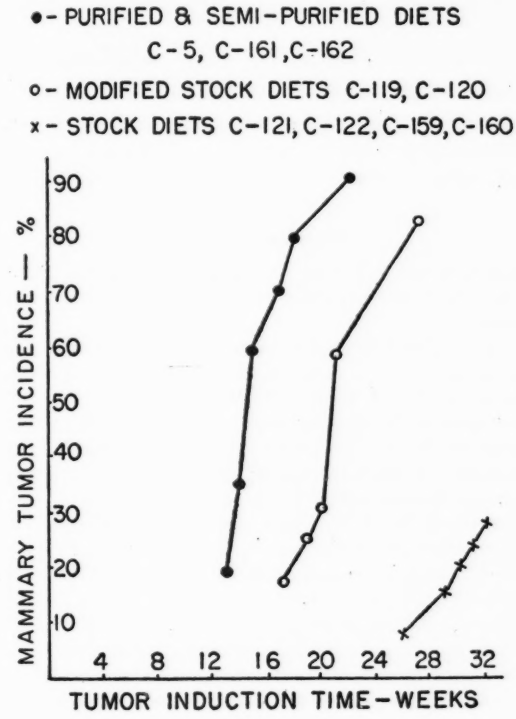

CHART 1.—Influence of diet on the induction time and incidence of mammary tumors in rats fed 2-acetylaminofluorene.

The modified stock diets (C-119, C-120 [Table 2]) failed to protect against tumor induction. However, it should be noted that the time required for mammary tumors to develop was 3–5 weeks longer than is usually required when the semi-purified diets are fed. Furthermore, the survival period for rats on diets C-119 and C-120 was considerably longer than that observed when the more purified diets were used.

The stock diets (C-121, C-122, C-159, and C-160) gave considerable protection against mammary tumor development. Of the 24 animals receiving these diets for 34 weeks or longer, only 8 developed mammary tumors (33 per cent incidence). Furthermore, the induction time for mammary tumors and also for ear duct tumors was increased significantly. The induction period for mammary tumors ranged from 28 to 31 weeks, a longer period than the average survival of rats fed 2-acetylaminofluorene in semi-purified diets. These results on mammary tumor incidence and induction time are graphically illustrated in Chart 1.

Further evidence of the protective action of stock diets against the carcinogenic action of 2-acetylaminofluorene is apparent from the data on animals that were removed after a 34-week experimental period (Table 2). At this time, half of the animals on diets C-121 and C-122 (six out of twelve) were still tumor-free. The tumor incidence increased when animals were continued on experiment for longer periods (diets C-159 and C-160), with only one animal in twelve remaining tumor-free during an experimental period of 42 weeks.

The data support at least a tentative conclusion that natural food ingredients afforded some protection against liver tumor induction. The incidence of liver tumors was only 17 per cent (4 of 24 rats) in the rats sacrificed after a 34-week experimental period (diets C-119, 120, 121, 122). In contrast, 31 of 47 rats (66 per cent incidence) receiving the semi-purified diets had developed liver tumors in an average survival period of 27 weeks.

A high incidence of ear duct tumors and an increased incidence of liver tumors eventually resulted in the animals that were continued on stock diets (diets C-159, C-160) until they died (42 weeks).

DISCUSSION

From the data presented, it is obvious that natural foodstuffs afford considerable protection against the carcinogenic action of 2-acetylaminofluorene in rats. In contrast to the results obtained with semi-purified diets, the natural foods also permitted better survival and prolonged the induction time for mammary, ear duct, and probably also liver tumors.

Many investigators who have studied the carcinogenicity of 2-acetylaminofluorene in rats have used diets composed of natural foodstuffs (1, 3, 4, 10, 12, 15).

Wilson and associates (16) noted that supplements of cod liver oil, dried brewer's yeast, and wheat germ did not influence the tumors produced or the tumor induction time in rats that received 2-acetylaminofluorene in a diet composed of corn, linseed oil cake meal, and casein. Later, it was reported by Wilson and DeEds (14) that the above diet afforded considerably less protection against the carcinogenic action of 2-acetylaminofluorene than did a commercial dog chow meal (Purina). Bielschowsky (2) reported that the development of cancers in rats induced by 2-acetylaminofluo-

rene could be significantly delayed by supplementing a diet of bread and skim milk with 15 per cent of yeast.

In addition to the published data from this laboratory, results by other investigators have been reported on the use of purified or partially purified diets in studies with 2-acetylaminofluorene. Harris (11) concluded that variations in the dietary protein level or supplements of liver extract to purified diets had no appreciable effect upon the production of liver tumors by this carcinogen. Miller and associates (12), in comparing the activities of various analogs of 2-acetylaminofluorene in rats, noted much better survival when crude diets were fed than when purified diets were fed. Morris (13) also found that rats survived better and developed more tumors when purified diets were used containing 18–24 per cent of protein than when the carcinogen was fed in diets containing only 12 per cent of protein.

An unusual dietary effect in 2-acetylaminofluorene carcinogenesis was reported by Dunning and associates (5). These investigators observed bladder cancers in 100 per cent of rats on a purified diet containing tryptophan-free casein hydrolysate supplemented with DL-tryptophan.

A review of the literature thus emphasizes that, although there is some conflict of opinion, the evidence suggests there is an influence of diet on 2-acetylaminofluorene carcinogenesis.

These experiments appear to be the first in which a relatively detailed comparison of purified food ingredients and natural foods has been made in regard to their influence on the action of 2-acetylaminofluorene. The results indicate that purified or semi-purified diets are preferable to diets of natural foodstuffs for studies in which a rapid and uniform production of tumors is desired. The results further suggest that, insofar as this study has progressed, none of the dietary ingredients seem to be responsible for any particularly striking anti-carcinogenic properties. However, a combination of all the ingredients commonly used in stock diets afforded much better protection against tumors than did semi-purified diets.

SUMMARY

When weanling rats of either the AES or the Sprague-Dawley strain were fed semi-purified diets containing 0.03 per cent of 2-acetylaminofluorene, they survived about 27 weeks, developed mammary tumors in over 90 per cent of cases in 13 to 22 weeks, and developed ear duct and liver tumors in 50–60 per cent of cases. Rats of the Sprague-Dawley strain appeared to be particularly susceptible to mammary tumor induction.

Rats fed the same level of the carcinogen on a diet of natural foodstuffs (stock diets) were still free of visible tumors up to the 28th week, on the average. Eventually, mammary tumors developed in 8 of 24 animals on such diets (33 per cent incidence). Survival on such diets was extended to 42 weeks, with 10 of 12 rats developing ear duct tumors and 7 of 12 rats developing liver tumors before death occurred. One of the 12 rats remained tumor-free throughout the experiment.

Modified stock diets produced results intermediate between those obtained on semi-purified and stock diets.

The results emphasize that semi-purified or purified diets are preferable to diets of natural foodstuffs for the early and consistent production of mammary and other type tumors in young female rats.

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Studies on Methylcholanthrene Induction of Tumors in Scorbutic Guinea Pigs*

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Warren and Gates (2) attempted a study of the effects of ascorbic acid deficiency in the induction of tumors in guinea pigs injected subcutaneously with 1,2,5,6-dibenzanthracene in lard. The animals were maintained on a scorbutic diet (Howe-Wolbach) with just sufficient orange juice to prevent death from scurvy. Of 30 animals used, one animal developed a lesion consisting of proliferative granulation tissue but not regarded as neoplastic. The authors felt that the results were of little value, since only a few of the animals survived beyond 100 days. The present communication reports the results of studies on the induction of tumors by methylcholanthrene administered subcutaneously to guinea pigs subjected to recurrent sublethal periods of scurvy. Morphologic and histologic studies of the tumors induced are reported elsewhere (1).

MATERIALS AND METHODS

Ninety-eight young but fully mature male and female guinea pigs averaging 300 gm. in weight were purchased from commercial breeders in St. Louis. Methylcholanthrene was dissolved in sesame oil, 10 mg/cc, and injected into the subcutaneous tissue of the axilla of the right foreleg. All animals received 30 mg. of methylcholanthrene in doses of 10 mg. at intervals of 2 weeks. The animals were divided into a test or deficient group of 39, a control group of 28, and an inanition group of 31.

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Scurvy was produced in the test group by feeding rabbit chow checkers prepared by the Purina Mills Company of St. Louis. The constituents of this ration are soybean oil, wheat germ meal, corn germ meal, alfalfa meal, ground oats, corn meal, gray wheat middling, molasses, calcium carbonate, iodized salt, and riboflavin concentrate. The exact amount of each constituent was not available from the manufacturer. Chemical analysis of rabbit chow checkers is shown in Table 1.

TABLE 1

CHEMICAL ANALYSIS OF RABBIT CHOW CHECKERS*

Constituent	Amount
Protein	17.50 per cent
Fat	3.60
Fiber	15.00
Ash	6.00
Water	10.00
Nitrogen free extract	47.00
Calcium	1.10
Phosphorus	0.42
Magnesium	0.18
Potassium	0.90
Soluble chloride as NaCl	0.90
Iron	2.75 parts per million
Copper	12.00 parts per million
Cobalt	0.05 parts per million
Manganese	100.00 parts per million
Carotene	3.00 parts per million
Vitamin D	2 U.S.P. units per gram
Ascorbic acid	0.00 parts per million

* This analysis was carried out by the laboratories of the Purina Mills Co. and kindly made available to the authors.

Animals fed this diet develop characteristic clinical signs of scurvy within 14 days and die in from 21 to 25 days. In the test group of animals the deficient diet was employed for periods of 14 days only, in order for the animals to live for a sufficient period to allow the carcinogen to produce tumors. The test animals lost an average of approximately 30 gm. during the 14-day deficiency period, but this weight was quickly regained when lettuce was returned to the diet. Following a recovery period with lettuce supplementation of from 10 to 30

days, another period of 14 days of the deficient diet was employed, etc. Control animals were fed rabbit chow checkers and lettuce ad libitum.

The inanition effect produced by the scurvy was controlled by simply restricting the amount of rabbit chow checkers in the diet of the inanition group, while ascorbic acid was supplied by subcutaneous injections of 5 mg. in normal saline every other day. By this procedure the animals were selectively starved so that they lost approximately the same amount of weight as the animals

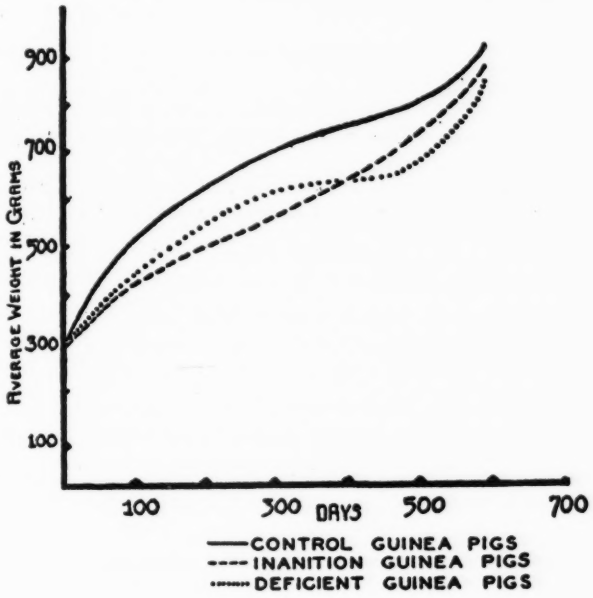

CHART 1.—Composite weight curves

in the deficient group. In the inanition control group ascorbic acid was used rather than lettuce, since this vegetable contains other factors.

RESULTS AND DISCUSSION

The first tumor appeared after 170 days, at which time 73 of the original 98 animals were living. Of these, 39 developed tumors. The experiment was terminated after 588 days, at which time the final fatality occurred in the deficient group, and a total of only 2 animals were living. Unfortunately, exact data are not available, but it is believed that there were no significant differences in longevity in animals not developing tumors in the three groups. It would follow that tumor incidence was similar in all groups. Composite weight curves of the groups are given in Chart 1.

Table 2 shows the time of appearance of tumors in the 3 groups, with a histologic classification. Statistical analysis of the results showed that the difference in time of appearance of tumors between the deficient group and the control group was highly significant ($P < 0.01$). On the other hand, the difference in time of appearance of tumors between the deficient group and the inanition group was not significant (P = approximately 0.15), nor was the corresponding difference between control and inanition groups (P = approximately 0.07) significant.

TABLE 2

INDUCED TUMORS ARRANGED IN ORDER OF APPEARANCE

Animal	Sex	Time of appearance in days: Deficient group	Control group	Inanition group	Type of tumor
30D	F	170			Liposarcoma
2D	F	174			Osteosarcoma
15I	M			175	Osteosarcoma
33D	M	177			Osteosarcoma
51D	M	177			Osteosarcoma
16I	M			179	Osteosarcoma
6D	M	180			Liposarcoma
48D	M	183			Fibrosarcoma
19I	M			183	Fibrosarcoma
50D	M	205			Liposarcoma
61I	F			215	Fibrosarcoma
46D	F	217			Osteosarcoma
62I	M			218	Osteosarcoma
66I	F			230	Fibrosarcoma
56D	M	233			Fibrosarcoma
12D	M	275			Liposarcoma
38N	M		284		Fibrosarcoma
41D	F	285			Fibrosarcoma
65N	M		290		Fibrosarcoma
68N	M		296		Fibrosarcoma
15D	F	310			Liposarcoma
58I	M			313	Fibrosarcoma
63I	F			333	Liposarcoma
68N	F		335		Fibrosarcoma
19D	F	336			Osteosarcoma
37D	M	340			Fibrosarcoma
8N	M		340		Fibrosarcoma
11I	F			353	Liposarcoma
5N	M		354		Fibrosarcoma
34D	M	368			Liposarcoma
71N	F		368		Osteosarcoma
60N	F		371		Fibrosarcoma
2N	M		380		Liposarcoma
67I	F			459	Fibrosarcoma
52I	M			479	Liposarcoma
36I	M			499	Fibrosarcoma
70N	M		543		Osteosarcoma
69N	M		563		Liposarcoma
21N	F		588		Liposarcoma
Average number of days for production of tumors:		242	393	303	

It cannot be assumed that ascorbic acid deficiency alone was responsible for the significant earlier occurrence of the tumors in the test group. During their many inter-scorbutic periods these animals received lettuce, which contains many factors other than ascorbic acid. The validity of this conclusion is borne out by the fact that there was no significant difference in time of appearance of tumors in the deficient group and the inanition group. In other words, intermittent ascorbic acid

deficiency broken by lettuce supplementation gave results similar to those obtained with comparable inanition but with constant and adequate ascorbic acid administration.

It is not possible, on the other hand, to conclude that ascorbic acid deficiency does not influence tumor development. Such an effect could conceivably have been neutralized by other factors in the lettuce used in the test group, or the intermittent ascorbic acid administration (in the form of lettuce) may have prevented the appearance of an effect significantly different from that produced by general inanition.

SUMMARY

Recurrent periods of scurvy, interspersed with periods of lettuce supplementation sufficient to prevent death, resulted in a significant shortening of the time of appearance of methylcholanthrene-induced tumors in guinea pigs. Comparable general inanition with constant ascorbic acid administration showed an intermediate time of appearance of tumors which was statistically not significantly different from the time of appearance in either the test group or the controls. Under the experimental conditions utilized in these experiments it was not possible to conclude that ascorbic acid deficiency affected methylcholanthrene carcinogenesis. On the other hand, a possible role of ascorbic deficiency has not been excluded.

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The Influence of Blockage of the Nipples on the Occurrence of Hyperplastic Nodules in the Mammary Glands of C3H Mice*

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It has been shown (2) that, in those DBA breeding females in which the nipples on the right side were blocked, an increased number of mammary tumors occurred at a younger age on the blocked right side, as compared to the intact left side. It was thought that milk stagnation was responsible for the increased tumor incidence.

Several investigators (1, 3) observed that mammary glands of high tumor strain mice contained hyperplastic alveolar nodules considered to be precancerous lesions. Huseby and Bittner (4) found that the occurrence of these lesions was dependent on the same three factors that are etiologically important for the development of mammary cancer—namely: genetic susceptibility, hormonal stimulation, and the presence of the milk agent. They observed that the lesions were uncommon in the low tumor lines irrespective of which of these "primary" factors was lacking.

In the C3H strain the mammary tumor incidence is almost as high in the virgin as in the breeding females. Since in virgin females there are no striking changes in the glands due to functional variations (pregnancy, lactation, regression), the hyperplastic nodules can be distinguished more easily and with greater certainty. In the present investigation virgin C3H females were used. The object of the investigation was to determine whether blocking the nipples would have any effect on the number of hyperplastic alveolar nodules in C3H virgin females possessing the milk agent and in those lacking it.

MATERIALS AND METHODS

The animals used were the inbred C3H/Fe mice possessing and the C3Hb/Fe mice lacking the milk agent. The milk agent-free line of mice was developed from the C3H/Fe line in the following way. Each of the nipples of an adult C3H female was drawn out with a pair of fine forceps and cut at the base. This resulted in the formation of scar tissues which blocked the main ducts. The female was mated with her brother, and the resulting new-born young that could not have received any milk from their mother were nursed by a C57 black foster-mother. From this litter our milk agent-free line was subsequently established and is designated as C3Hb/Fe.

In the experiments reported here, the blockage of the nipples was accomplished as described above, by cutting the nipples of weaning age animals. In half of the animals all the glands of the right side and in the other half all the glands of the left side were blocked. A few animals were killed at 7 months of age, the majority at 8 and 9 months. The skin with the mammary glands on it was carefully removed from all animals, spread out, and flooded with fixative. Dehydration by alcohol and removal of the fat by xylene made the glands easily visible. They were examined and the hyperplastic nodules counted with the aid of a dissecting microscope. Sample glands were dissected from the skin prepared and stained for study as gross mounts. A few of the hyperplastic nodules were sectioned for more detailed study.

RESULTS

C3H/Fe mice possessing the milk agent.—Thirty-one animals in which all mammary glands on the right sides were blocked were examined, and hyperplastic nodules were found in twenty of them. Fifty-six nodules occurred on the blocked right sides and 38 on the intact left sides. Three animals had mammary tumors at 9 months of age; two of them on the right side and one on the left side (Table 1).

Thirty-one animals in which all mammary glands on the left sides were blocked were examined, and hyperplastic nodules occurred in sev-

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enteen of them. Thirty-six nodules were found on the left blocked sides and nineteen on the intact right sides. One mammary tumor was found on the left, blocked side of an 8-month-old animal (Table 2).

Combined data collected on the 62 animals described above reveal that considerably more nodules were found on the blocked nipple sides (92) than on the intact sides (57). This difference is statistically significant P = 0.001. The number of nodules on the right side (75) and those on the left side (74) were almost equal (Table 3).

C3Hb/Fe mice lacking the milk agent.—Sixty-two animals were examined, in half of them all the right and in half of them all the left mammary glands were blocked. There were no hyperplastic nodules in any of the glands of these animals.

In degree of development and pattern of duct structure of the mammary gland, the C3Hb/Fe (milk agent-free) animals showed no significant difference from the C3H/Fe animals (possessing milk agent) (Figs. 1 and 2).

TABLE 1

RESULTS OF THE EXAMINATION OF THE MAMMARY GLANDS OF C3H/FE MICE WITH RIGHT SIDE BLOCKED

No. of mice	Age (months)	No. of mice with nodules	No. of mice with nodules on: R. side	L. side	No. of nodules on: R. side	L. side
1	7	1	1	0	1	0
21	8	11	11	8	29	20
9	9	8	7*	8*	26	18
Total 31		20	19	16	56	38

* Two mice had mammary tumors on the right side and one had a mammary tumor on the left side.

TABLE 2

RESULTS OF THE EXAMINATION OF THE MAMMARY GLANDS OF C3H/FE MICE WITH LEFT SIDE BLOCKED

No. of mice	Age (months)	No. of mice with nodules	No. of mice with nodules on: R. side	L. side	No. of nodules on: R. side	L. side
1	7	1	0	1	0	4
20	8	9	5	8*	17	24
10	9	7	1	5	2	8
Total 31		17	6	14	19	36

* One mouse had a mammary tumor on the left side.

DISCUSSION

It is difficult to explain why the blocking of the nipples should affect the number of precancerous hyperplastic nodules. Our animals were virgin females; therefore, the glands were not functioning, and milk stagnation could not account for the increased number of nodules. The mammary tumor incidence of the C3H mice is high in the virgin females, showing that milk secretion perhaps is not a necessary step in tumor development. Huseby and Bittner pointed out "that although a close correlation exists between these hyperplastic areas and cancer, the tumor incidence of a strain may not be strictly paralleled by the number of precancerous lesions developing in the mammae." The possibility exists, however, that, because more of these hyperplastic nodules are present on the blocked side, there is a greater chance for the later development of mammary tumors on this side.

The development of the hyperplastic nodules in our experimental animals depended on the presence of the milk agent. The C3Hb/Fe mice had the same genetic susceptibility, presumably the same hormonal stimulation, and lacked only the milk agent. This lack was evidently sufficient to prevent the occurrence of hyperplastic nodules.

Structurally, the hyperplastic nodules are composed of clusters of small alveoli which must arise from and indeed show increased mitotic activity. They are therefore localized growth centers that are evidently not under the same control as the rest of the gland. At present, there is no explanation for the observed fact that the presence of the milk agent influences the development of such growth centers. Similarly, there is no explanation for the observed fact that blocking the nipples in some way increases the probability for the development of hyperplastic nodules.

TABLE 3

COMBINED RESULTS OF TABLES 1 AND 2

No. of mice	Age (months)	Total no. of mice with nodules	No. of mice with nodules on: Blocked side	Unblocked side	No. of nodules on: Blocked side	Unblocked side	No. of nodules on: R. side	L. side
2	7	2	2	0	5	0	1	4
41	8	20	19	15	53	37	46	44
19	9	15	12	10	34	20	28	26
Total 62					92	57	75	74

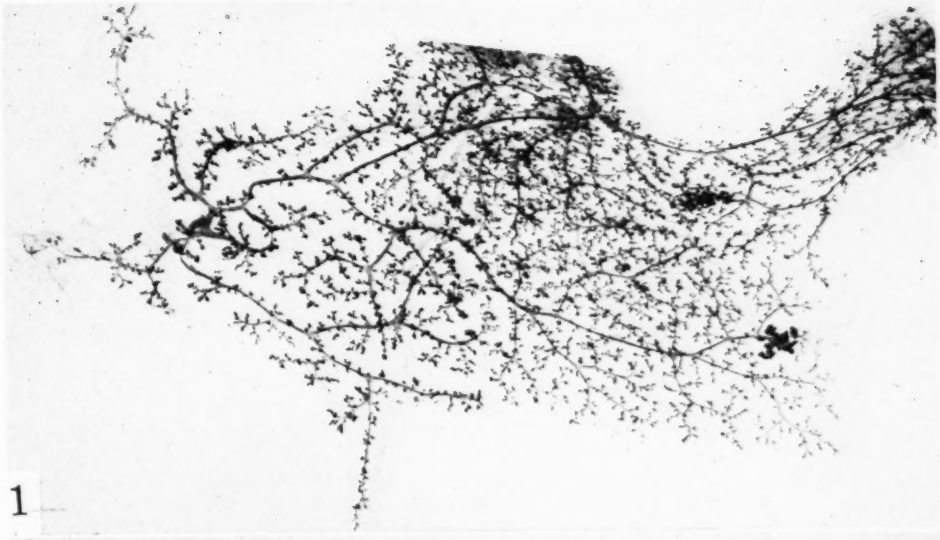

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FIG. 1.—Whole mount preparation of the blocked right third mammary gland of an 8–month–old C3H/Fe female. Note the hyperplastic nodules. ×4.5.

FIG. 2.—Whole mount preparation of the blocked left third mammary gland of an 8–month–old C3Hb/Fe female. Hyperplastic nodules are not present, but in degree of development this gland is similar to the one shown on Figure 1. ×4.5.

SUMMARY

The mammary glands of 62 virgin females of the C3H/Fe strain possessing the milk agent and an equal number of C3Hb/Fe females lacking the milk agent were blocked on one side by cutting their nipples at weaning age. The animals were killed at 7–9 months of age. The numbers of hyperplastic nodules found on the blocked and unblocked sides were compared.

In the C3H/Fe mice possessing the milk agent more hyperplastic nodules were found in the mammary glands of the blocked sides (92) than on the unblocked sides (57). This difference is statistically significant ($P = 0.001$).

In the C3Hb/Fe mice lacking the milk agent hyperplastic nodules were not found in the mammary glands of either on the blocked or unblocked sides.

ACKNOWLEDGMENTS

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Neoplasia Induced in Rat Embryos by Roentgen Irradiation*

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During the course of studies on the over-all effects of irradiation on embryonic development, it was observed that tumor-like growths occurred in the head region of rats exposed to x-rays on the ninth day of gestation (6). Although all were probably of neural origin, many of these growths retained no connection with the brain. Disordered growth in the central nervous system, including malformations and localized aberrations of brain tissue known as "rosettes," has been repeatedly described by several investigators following irradiation of mammalian embryos (1–5, 7). The present authors know of no other instance, however, in which discrete foci of abnormal growth have been reported as developing independently of the nervous system in animals irradiated during the embryonic period.

This communication describes the method of production, the incidence, and the growth characteristics of such independent tumors in embryonic and newborn rats. Presumptive evidence bearing on the manner of origin and the ultimate fate of these growths is also presented and discussed.

METHODS

A method whereby selected rat embryos can be exposed to x-rays without exposing the mother or the remaining embryos has been described in detail elsewhere (7). Only the essential points of the procedure will be given here. Female rats of the Wistar albino strain were considered to have begun gestation at 9 A.M. of the morning on which sperm was found in the vaginal smear, after the females had been caged with males of the same strain on the preceding night. The embryos were regarded as 1 day of age 24 hours later. Nine days (approximately 216 hours) after the estimated beginning of gestation the pregnant animals were anesthetized with Nembutal, and the ventral abdominal wall was opened by a midline incision. One uterine horn was brought to the surface of the incision, stabilized by means of moist gauze packs, and shielded with lead plates in such manner that only three to five implantation sites were left unshielded. Additional lead plates were used to insure complete shielding of the mother and the remaining implantation sites. After exposure of the unshielded embryos to the desired dosage of x-rays, this uterine horn was replaced, and the second horn was brought to the surface and carried through similar manipulations, except that no embryos in the second horn were irradiated. Before the latter was restored to the abdominal cavity and the incision closed, the number and location of all embryos in both horns were recorded on a diagram to facilitate later identification of irradiated and nonirradiated embryos.

Doses of 25, 50, 100, or 200 r (in air) were given at a single exposure to the unshielded embryos. A therapeutic x-ray machine calibrated with a standardized Victoreen r-meter was used. The beam was that produced by the factors: 85 kvP, 10 ma, and 1.0 mm. aluminum added filter. Further characteristics were: HVL of 2.0 mm. of aluminum, and the 1.0-cm. depth dose exactly equivalent to the air dose as measured in a prestwood phantom. The target object distance varied between 10 and 20 cm., and the duration of exposure never exceeded 2 minutes.

After post-irradiation intervals ranging from 1 to 12 days (term), the mothers were killed by decapitation, and the irradiated and nonirradiated embryos identified by use of the diagram made at the time of exposure. All embryos were fixed in Bouin's fluid, transferred to 70 per cent alcohol, and subsequently examined for externally visible abnormalities prior to imbedding in paraffin. The irradiated embryos, together with one or two of their nonirradiated siblings, were serially sectioned and stained with hematoxylin and eosin.

OBSERVATIONS

When examined externally 4–8 days after exposure to the higher doses of x-rays (100 and 200 r), many of the embryos exhibited over the

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head region opaque spots that were clearly visible through the ectoderm (Fig. 1, *A* and *B*) under low-power stereoscopic magnification. In histologic sections these appeared as dense clusters of cells situated in various relationships to the normal tissue layers of the head. They were most frequently associated with the mesencephalon, either attached to it (Fig. 2, *A*, *B*, and *C*) or lying free in the surrounding mesenchyme (Fig. 3, *A*, *B*, and *C*), but they were seen in relation to all parts of the brain rostral to the pontile flexure. Occasionally, such clusters of cells were more remote from the brain and lay in contact with the ectoderm (Fig. 4). Since the masses did not correspond in appearance or location to any structure normally seen in rat embryos and since their pattern of growth was obviously independent of that in any normal structure, they were considered to be neoplastic.

General features.—Histologically, the tumors consisted of closely packed groups of cells which bore noticeable resemblance to contemporary cells in the walls of the developing neural tube. After the third post-irradiation day, their size and the amount of mitotic activity they displayed varied considerably. Their shape and internal structure also were subject to variation, but the growths tended to be spherical and usually exhibited a radial arrangement of cells about a central point or small vesicle, with the most centrally placed cells exhibiting the greatest mitotic activity (Figs. 2 and 3). In the smaller growths all cells were frequently involved in such a configuration, but in the larger ones the original focus was overlaid on the surface by varying numbers of randomly arranged cells (Fig. 3, *C*).

Incidence.—The incidence of embryonic tumors was directly related to the quantity of radiation received by the embryos (Table 1). Aberrant growths were extremely rare in nonirradiated animals: only 1 of the 34 control embryos was found to contain an abnormal cell cluster in any way resembling those found in the irradiated embryos. This animal had a small group of epithelioid cells isolated in the mesenchyme, simulating the tumors except for the lack of mitosis or other evidence of rapid growth. On the other hand, exposure to as little as 25 r of x-rays caused abnormal growths to appear in 20 per cent of embryos; and increased dosages resulted in an increased percentage of affected embryos, to the extent that virtually all animals receiving 100 r later developed one or more tumors. Not only did the percentage of affected animals increase with the dosage, but the average number of growths per affected animal was also increased in proportion to the dosage. For example, whereas 3 of 15 embryos receiving 25 r had an average of 1.3 growths, 48 of 49 embryos receiving 100 r had an average of 7.4 growths per affected animal.

Another factor influencing the incidence of these tumors was the age of the embryo at the time of irradiation. The high incidence described above was found only in animals irradiated on the ninth day of gestation. In a preliminary report (8) it was noted that abnormal growths were more prevalent in embryos irradiated on the ninth than on the tenth day. A more extensive comparison of the two groups has confirmed the earlier observation and indicates further that: in the group irradiated on the tenth day, tumors rarely occurred in animals receiving less than 100 r; no more than 30 per cent of embryos had growths even after larger dosages; the number of growths rarely exceeded one or two per affected animal; and the tumors remained relatively small in size. An additional group of embryos has since been exposed on the eighth day to doses of x-rays comparable to those used on the ninth and tenth days, and preliminary study indicates that neoplasia in these animals is rare or absent, no growths having been found to date. It is clear, therefore, that x-radiation of rat embryos on the ninth day of gestation is much more effective in producing this type of embryonic tumor than is treatment with similar dosages on the eighth or tenth days.

TABLE 1

ABNORMAL GROWTHS IN CONTROL AND IRRADIATED EMBRYOS*

	Controls	25 r	50 r	100 r	200 r
Total no. of embryos	34	15	32	49	9†
Per cent of embryos affected	3	20	59	98	100
Av. no. per affected embryo	1	1.3	2.7	7.4	11.3

* Based on embryos removed 2–6 days after irradiation.
† Does not include many embryos killed by this dosage.

Types, based on their location.—Although histologically similar, the tumors varied in their relationship to the normal tissue layers of the head. Since their fate was found to be influenced by such relationships, the tumors have been classified in five categories based on their location. Table 2 presents the relative incidence (as the percentage of the total number of growths) of the five types as they occurred in embryos removed 2, 4, 6, or 8 days after irradiation with 100 r. Three of the types were in direct continuity with the tissues of the neural tube: (*a*) the "polypoid" growths which protruded into the brain cavity from the ependymal surface; (*b*) the "rosettes" or intramural growths which lay within the wall of the neural tube, recognizable only by virtue of their

peculiar organization; and (*c*) the "attached" growths which projected from the outer or marginal surface of the neural tube into the surrounding mesenchyme. Not directly related to the brain were: (*d*) the "isolated" growths which lay free within the mesenchyme between the neural tube and the ectoderm, and (*e*) the "cutaneous" growths which were also within the mesenchyme but were in contact with the ectoderm covering the head.

It is apparent from the percentages presented in Table 2 that the relative numbers of the different types of tumors changed as the interval following irradiation lengthened. Rosettes were not seen prior to the fourth day and were rare on the fifth day, after which they were seen in moderate numbers. The abnormal cells from which the rosettes arose were undoubtedly present prior to the fourth day but became distinguishable from the surrounding medulloblasts only after the distinctive radial arrangement of cells began to appear. The situation was reversed for cutaneous growths which were frequently seen following the shorter intervals but were not found after the sixth post-irradiation day. The manner of disappearance of these will be described in another section. Polypoid growths were seen infrequently at all intervals.

TABLE 2

RELATIVE INCIDENCE OF TUMORS IN VARIOUS LOCATIONS IN AND ABOUT THE BRAIN
(See text for explanation)*

POST-IRR. INTERVAL (DAYS)	NO. OF EMBRYOS STUDIED	INCIDENCE OF EACH TYPE AS PER CENT OF TOTAL GROWTHS				
		Polypoid	Rosette	Attached	Isolated	Cutaneous
2	13	8	0	37	38	17
4	18	2	1	19	57	21
6	22	6	14	20	56	4
8	8	6	18	29	47	0

* Based on animals receiving 100 r.

At all ages the majority of growths fell into the "attached" and "isolated" categories (Table 2). These occurred with about equal frequency on the second post-irradiation day, but by the third day the isolated tumors were found in somewhat greater numbers, the increase being of about the same magnitude as the decrease in attached growths. Beyond the third day the two types remained in an approximate 1:2 ratio. As will be discussed below, the shift in ratio after the second day may indicate that some of the attached growths broke away from the neural tube to lie free in the mesenchyme, subsequently to be classified as isolated growths.

Origin of the growths.—At the time of irradiation on the ninth day of gestation, the embryos were at the primitive streak stage of the egg cylinder, meaning that the embryonic body consisted of nothing more than the three primary germ layers: ectoderm, mesoderm, and endoderm, with practically no differentiation within any of the three. Since the neural plate was not recognizable, there was no morphological distinction between neural and cutaneous ectoderm, although the fact that the primitive node and streak were beginning admits the probability that the cephalic part of the neural primordia was already determined in the sense of embryonic induction. Further speculation along these lines is unwarranted; it only need be emphasized that no definitive tissue layers were recognizable at the time the embryos were irradiated.

Foci of abnormal growth were not seen in embryos removed 24 hours and less after exposure to x-rays, although general retardation of growth was apparent following the higher dosages. Embryos removed 48 hours after the exposure, however, bore small but easily recognizable clusters of cells in several abnormal locations. Most often they protruded into the mesenchyme from the outer surface of the neural tube or were isolated islands within the mesenchyme, but the other types, excepting the rosettes, were also present (Table 2). Regardless of location, all these early growths consisted of cells that were identical in appearance.

This histologic similarity is the basis for assuming that the abnormal cells arose from a common source—that is, from cells which had the same developmental potentialities before irradiation. To state precisely what this potentiality might have been (whether to form neural, cutaneous, or vascular or other mesodermal tissues) is not possible, although there are several lines of evidence indicating that the abnormal cells may have arisen in common with the neural primordia. First, it should be recalled that three of the five tumor types (the polyps, the rosettes, and the attached growths) remained intimately associated with the neural tube in later development. Secondly, all the tumors were not only intrinsically similar but also bore pronounced histologic resemblance to the tissues in the normally developing neural tube. Furthermore, the differentiation later observed in many of the tumors tended to parallel in degree and time that occurring normally in the neural tube.

If it is assumed that all abnormal growths arose from cells originally contained in the neural ectoderm, the manner in which the isolated and the cutaneous growths separated from the parent

layer requires explanation. On the second day after irradiation both of these were already present as discrete types; but, as indicated in Table 2, their incidence was lower than after longer intervals, particularly with the isolated growths. In view of this, and the fact that the total number of all tumors increased between the second and third days, it is apparent that new tumors were arising or becoming recognizable during this period. Among the numerous growths seen protruding into the mesenchyme from the outer surface of the neural tube (Fig. 5, *A*, *B*, *C*, and *D*) on the second day were many which retained only an attenuated connection (Fig. 5, *D*). Continued outward migration of the main mass of abnormal cells or the development of mechanical stresses following unequal growth within the head region could have broken or dislodged the slender pedicle, thus converting an attached to an isolated growth. The relationship depicted in Figure 5, *B*, in which a mass of cells protruding from the neural tube is in contact with the ectoderm, was also observed with some frequency on the second day. Retention of the ectodermal contact but with subsequent loss of the connection to the neural tube, as postulated above, would exemplify a possible manner of origin for the cutaneous growths. The further possibility that cutaneous growths arose directly from cutaneous ectoderm cannot be denied; however, the fact that they usually appeared to be contiguous rather than continuous with the ectoderm suggests that they arose elsewhere and only secondarily come into apposition with ectoderm.

Growth of the tumors.—The small clusters composed of a few cells seen on the second day displayed varied capacities for growth as the embryo became older. Some grew little or not at all after the second day, others grew rapidly until the fifth or sixth day and then became atrophic, while still others continued to grow until the seventh or eighth day after treatment (Fig. 6, *A*). Precise measurements of rate of growth have not been attempted, although estimates of volume, based on diameters in section and appraisals of mitotic activity, have provided data for comparative purposes. Even these crude methods were sufficient to demonstrate that an increase in volume of several hundred per cent was not uncommon between the second and sixth post-irradiation days. The rate of increase in the most rapidly growing tumors, however, never exceeded, on a relative basis, that of many regions of the normally growing brain. Indeed, after the sixth day it was rare to find a tumor containing as many mitotic figures as a comparable area of brain tissue. By the eighth day there were few tumors that gave any evidence of continued proliferation, the majority seemed to have become static or actually atrophic (Fig. 6, *B* and *C*).

The rate and duration of growth were to a large extent related to the type of tumor—that is, the location of the tumor with respect to the usual tissue layers of the head. Attainment of large size was limited to two types, namely, those attached to the outer surface of the brain and those isolated within the mesenchyme, and of the latter only those in close proximity to the brain. Isolated growths in more remote mesenchymal areas generally ceased growing early. Cutaneous growths rarely exceeded the small size seen on the second day, and the exceptional ones that did reach appreciable size (Fig. 4, *B*) generally showed signs of disintegration by the fourth day post-irradiation. Typically on the fourth day they were small, contained pyknotic nuclei and were devoid of mitotic figures (Fig. 4, *A*); by the sixth day few remained; and after that none were found (Table 2). Polyps, although encountered in small numbers at all ages, generally did not grow larger than the size reached on the second or third post-irradiation day. It was impossible to determine the increase in the size of the rosettes, because the cells that may have been proliferated from the original focus were not sharply distinguished from the surrounding neural cells.

TABLE 3

REDUCTION IN THE NUMBER OF TUMORS AS THE POST-IRRADIATION INTERVAL INCREASED*

	Post-irradiation day							
	2	3	4	5	6	7	8	12
No. of animals studied	13	3	18	4	22	12	8	5
Per cent of animals affected	92	100	100	100	100	52	88	40
Av. no. tumors mors per affected animal	4.0	16.0	7.8	7.0	6.6	4.3	4.2	2.5

* Based on animals receiving 100 r.

Regression of the tumors.—After the third post-irradiation day the average number of growths per animal decreased steadily until term (Table 3), at which time very few recognizable ones remained. How this reduction was brought about is not entirely clear, although two processes have been observed which undoubtedly account for the disappearance of some of the tumors. On the third day after treatment a number of the smaller isolated growths were found to lack the compact organization typically seen at this time. In some the only change, aside from the loose arrangement of cells, was the lack of mitotic activity; but in

others the cells were more or less scattered throughout a limited region of mesenchyme (Fig. 7, *A* and *B*). In extreme instances such a region contained few recognizable tumor cells but much cellular debris and several large multinucleated cells (Fig. 7, *C*). The latter probably resulted from coalescence of degenerating cells, although the possibility of phagocytic activity has not been eliminated. This dispersion of cells, followed by cellular disintegration, was observed sufficiently often on the third and fourth days to account for much of the reduction in the number of tumors that occurred during this period.

After the fourth post-irradiation day, increasing numbers of tumors were affected by another type of regressive process which appeared to be atrophic in nature. The tumors became dense and shrunken with small distorted nuclei and no mitotic figures. Blood vessels were usually lacking in the vicinity of such atrophic growths (Fig. 8, *A* and *B*). Varying degrees of these changes were found, from mere cessation of mitosis to the extreme in which only a minute cluster of flattened cells remained, suggesting that the process was a progressive one. Atrophy did not affect all growths in the same animal at the same time; while some began to show such change, others were still proliferating rapidly (Fig. 9). A majority of the cutaneous growths became atrophic by the fourth day (Fig. 4, *A*); most of the smaller isolated growths were affected between the sixth and eighth days (Fig. 6, *C*), but the attached tumors resisted atrophy until after the eighth post-irradiation day. Few growths of any size were found in animals reaching term (only 40 per cent of individuals were affected, and these averaged only 2.5 per animal), and, since the remaining ones were small or moderate in size and obviously atrophic or static (Fig. 10, *A* and *B*), it is evident that all the larger ones had regressed.

Lack of blood supply appeared to be a factor in limiting growth and also probably played a part in initiating regressive changes in some instances. The most profuse vascular bed found anywhere in the head of a developing embryo is in the mesenchyme immediately adjacent to the outer surface of the brain. It was in precisely this location that the larger tumors developed (see Figs. 6, *A*, 9, and 11), either near or attached to the brain; and it was the growths so situated that showed the greatest capacity to resist regressive change. Conversely, cutaneous growths and those isolated in more remote portions of the mesenchyme were affected earliest, possibly because of the limited vascularity. Failure of the polyps to reach large size may also be attributed to inadequate blood supply; any vessels reaching them had first to pass through the entire thickness of the neural tube, itself a rapidly growing tissue which most certainly placed heavy demands on the local blood supply.

Differentiation.—All the tumors which attained more than the minimal size seen on the second and third days showed some tendency to differentiate—that is, to show specialized organization or structure. The earliest, as well as the most common change in this direction, was the radial arrangement of cells about a central point or vesicle, typically seen on the fourth day (Figs. 2 and 3). In some instances neither further growth nor differentiation occurred, and the tumor remained as a small vesicle or sphere. In those that continued to proliferate, however, the new cells piled up in random fashion about the organized center which generally persisted as the focus of mitotic activity. Evidence of true cellular specialization first became apparent on the fifth and sixth days, when many of the larger growths developed a relatively cell-free cortical zone histologically resembling the marginal layer of the developing brain (Fig. 11). Although no special methods to demonstate axis cylinders were employed, examination of such areas under high magnification left little doubt but that they were made up of cell processes similar to those known to be present in the marginal layer of the brain. The processes were not always confined to the surface of the tumors but were sometimes seen to extend as filaments or definite nervelike bundles out into the surrounding mesenchyme. It was occasionally possible to trace these outgoing processes to the brain, where they merged with the marginal layer; others simply ended randomly in the mesenchyme. Several of the smaller growths exhibited slender chainlike extensions of cells out into the mesenchyme (Fig. 12). In rare instances a tumor assumed cystic form, appearing as a large vesicle enclosed by a thin-walled layer of cells, some of which were vacuolated such as to suggest secretory activity (Fig. 13).

Malignancy.—The capacity of these tumors to destroy the animal seems to have been insignificant. Although the mortality rate rose steadily from the third post-irradiation day until term (Table 4), none of the deaths could be attributed directly to the tumors. Study of complete serial sections of many embryos and fetuses, together with careful dissection of several newborn animals, revealed that the tumors were very rarely of sufficient size or in such strategic location as to cause death of the animal. On the other hand, numerous malformations of the brain (see following section) and cardiovascular system were found, and these

were often of such nature as to be possibly incompatible with life during the late fetal and newborn periods. Considering the fact that virtually all animals exposed to 100 or 200 r had tumors when examined at earlier ages, it is surprising that so few indications of tumor growth were seen at term, and more so that they did not more often interfere with the development and survival of the fetus.

Other examples of disordered growth.—In addition to the discrete and localized foci of abnormal growth described above, embryos irradiated on the ninth day of gestation also contained other manifestations of disordered growth. Since these involved the major portion of an organ or an entire region, they were considered to be malformations and as such will be described in detail elsewhere. Nevertheless, the component tissues of such malformed structures often showed as little conformity to the rules of normal growth as did the tumors. It is not to say that all malformations have features in common with neoplastic tissues—indeed, very few of them do. Only malformations involving hyperplasia, in the sense of overgrowth, and paraplasia, in the sense of aberrant growth, are proposed as bearing some analogy to neoplasia. Regardless of the correctness of such an analogy, it was observed that the brain of irradiated embryos was often affected by true malformations of the hyperplastic and paraplastic varieties. These involved disordered growth in a more or less extensive section of the brain wall, most often taking the form of diverticula in which the entire thickness of the brain wall in a circumscribed region was acutely bulged outward (Fig. 14). Occasionally, large areas—e.g., an entire telencephalic lobe—appeared to be involved; and in the more extensive cases the affected wall of neural tube was generally pushed outward to the extent that it contacted the overlying ectoderm. When such contact occurred over a large area, with obliteration of the usual intervening mesenchyme and blood vessels, rupture to the exterior was frequently the result, followed by herniation and eversion of the affected region of brain (Figs. 15 and 16). This sequence of events was obvious when successively older irradiated embryos were studied. If the diverticulum or larger protruding mass of brain did not rupture, the overt appearance was that of encephalocele or, if extreme, hydrocephalus. Similar but generally less extensive defects were occasionally observed in the spinal cord. It must be emphasized that none of these anomalies were the result of failure of the neural tube to close, but rather were growth aberrations involving a circumscribed region in the wall of an originally intact neural tube.

TABLE 4

PERCENTAGE MORTALITY AT DIFFERENT INTERVALS AFTER IRRADIATION WITH 100 r

	Days			
	3 and 4	5 and 6	7 and 8	12 (term)
Control	5	10	6	14
Irradiated	9	23	25	35

DISCUSSION

It was concluded above that the cells which later gave rise to the tumors were, before irradiation, among those destined to take part in formation of the neural tube. In fact, after irradiation the tumor cells differed from those comprising the neural tube principally in their failure to conform to the organizational pattern of this structure. They contributed nothing to the process of organogenesis of the presumed parent tissue and often failed to remain within the normal confines of that tissue. Thus, although the tumor cells conformed with others of their generic stock in matters of morphology and subsequent differentiation, they had undergone some change which destroyed the propensity to fit into a common pattern of organization with other cells in the developing brain. There is little doubt but that this change was induced by irradiation.

In the light of the known principles governing the actions of radiation on living cells, there is nothing to contradict the belief that the tumors arose from single cells, the genic constitution of which had been altered by irradiation. In any population of cells that is irradiated, only certain ones are "hit," the percentage so affected being dependent upon the quantity of radiation used. The affected cells suffer chemical or structural change which may be promptly manifested in cell death or delayed mitosis, or the changes may be of a more subtle nature and not overtly manifest until some time later. In the latter case vital activities are not impaired, and the cell and its descendants continue to appear normal until some critical stage of structural or functional differentiation is reached. Then, however, these cells fail to follow the prescribed course, and only then does it become apparent that the factors controlling such potentialities (presumably genes) have been altered by the irradiation. Such a "somatic mutation" could have accounted for the failure of the tumor cells to take their usual place in the organization of the brain.

The assumption that the tumors arose from genetically altered cells in the neural ectoderm does not explain why they were later associated

almost exclusively with the cephalic part of the neural tube. The fact that they were induced much more readily by irradiation on the ninth day than on the eighth or tenth days can be attributed to the likelihood that the cells in the cephalic part of the neural tube were at a critical or particularly labile stage on the ninth day (see page 224). Such an explanation for the regional localization of the growths, however, seems improbable. Differentiation in the neural tube proceeds in cranio-caudal sequence; therefore, fluctuations in the time of irradiation would be expected to cause a shift in the segment of the neural tube affected. Such was not the case, either after the unavoidable variations of a few hours in timing gestation or after the intentional variation of one day, when embryos were irradiated on the eighth or tenth day. A few animals irradiated on the tenth day did develop small tumors, but these were also in the vicinity of the midbrain or forebrain, precisely the area of predilection after ninth-day irradiation.

A unique feature of the tumors was their tendency to undergo spontaneous regression. Those situated at some distance from the rich vascular bed about the brain may have lacked sufficient blood supply to sustain rapid growth. Those adjacent to or actually attached to the brain, on the other hand, appeared to be adequately vascularized, yet they also failed to continue rapid growth and became either atrophic or static. The reasons for this are obscure, although two possibilities seem worthy of mention. It has already been suggested that the tumor cells were genetically or metabolically altered by the irradiation. Perhaps the nature of this change was such as to lower the proliferative capacity of the cells or to render them somehow less able to "compete" with normal embryonic cells. Another possibility is that the embryonic organism itself developed some mechanism of defense by which neoplastic growth was selectively suppressed.

Most of the growths are known to have disappeared or at least regressed to an unrecognizable form between the sixth post-irradiation day and term. Although not present as visible tumors, there is the possibility that a few scattered and inconspicuous tumor cells remained. This raises an intriguing point for speculation. Could such remnants lie dormant for a period and then later somehow be stimulated to resume the embryonic type of rapid growth? If so, the "theory of embryonic rests" as regards the origin of neoplasms would receive important substantiation. This possibility is being investigated by allowing animals irradiated on the ninth day of gestation to reach term and then grow to maturity.

SUMMARY

Rat embryos exposed to x-radiation on the ninth day of gestation developed discrete tumor-like growths in and around the brain. The incidence of such tumors was directly related to the dosage of x-rays. They first appeared on the second day after irradiation and thereafter exhibited varying capacities for growth and differentiation. Some grew for 1 or 2 days, then disappeared as a result of dispersal of the cells; others grew rapidly until the fifth ar sixth post-irradiation day, then became atrophic; and still others continued to grow slowly until the seventh or eighth day, then became static or underwent atrophic regression. Few remained in newborn animals, and these were small and gave no evidence of proliferative activity. Although the tumor-bearing animals had a somewhat higher mortality than the nonirradiated controls, neither prenatal nor postnatal death could be attributed to the presence of the tumors.

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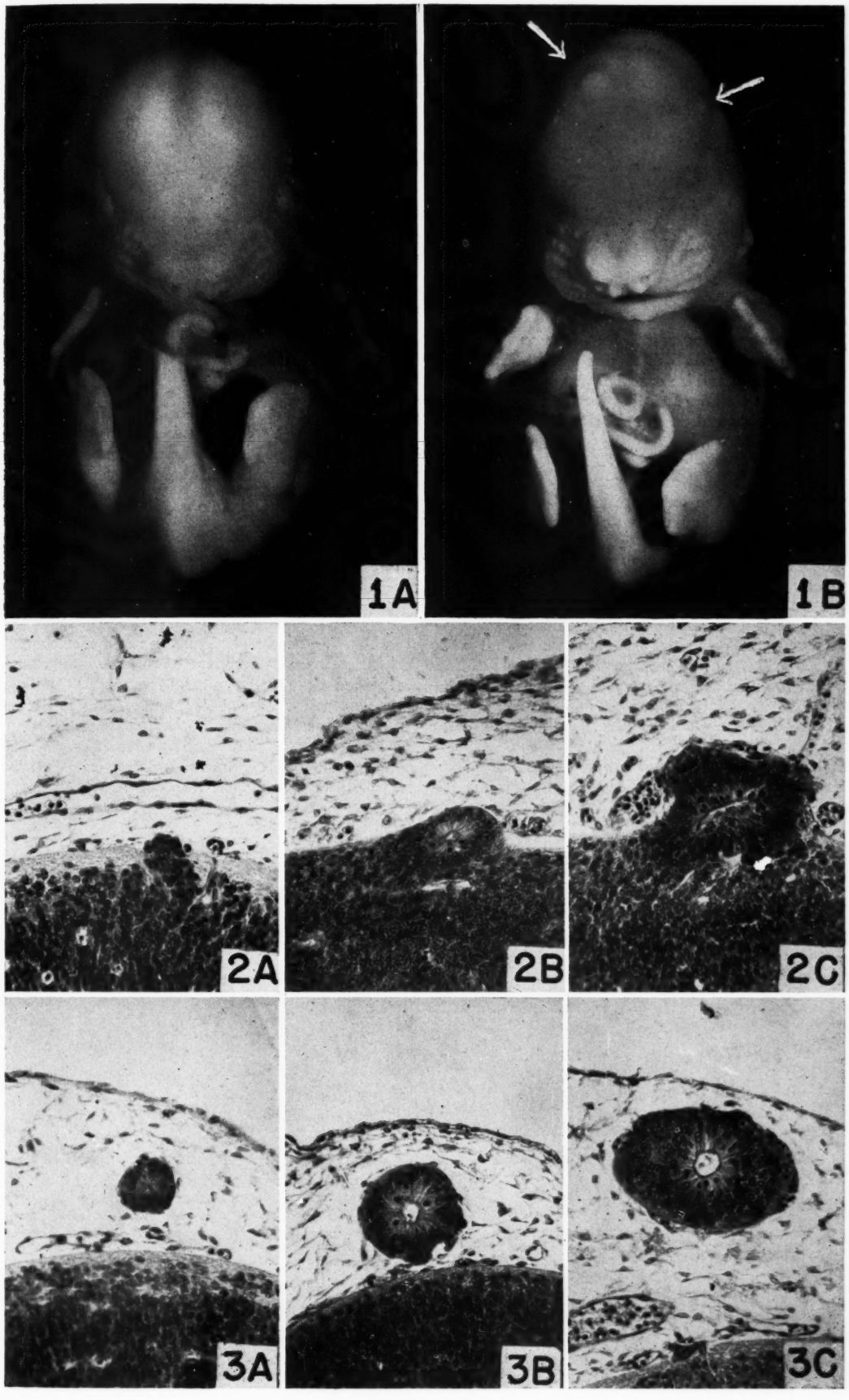

1A
1B
2A
2B
2C
3A
3B
3C

FIG. 1.—Two embryos removed from the same pregnancy on the fifteenth day of gestation. *A* was shielded with lead plates during irradiation and is normal. *B* was exposed to 100 r on the ninth day and allowed to live for 6 days thereafter. Opaque spots are visible underneath the ectoderm of the head, as indicated by arrows. ×9.

FIG. 2, *A*, *B*, and *C*.—Abnormal growths of various sizes and shapes attached to the outer (marginal) surface of the developing brain. From embryos removed 4 days after irradiation with 100 r on the ninth day of gestation. ×160.

FIG. 3, *A*, *B*, and *C*.—Abnormal growths lying free within the mesenchyme, between the neural tube and the ectoderm. Many of the tumors at this stage of development exhibited a conspicuous radial arrangement of cells about a central point or vesicle (see *B* and *C*). From embryos removed 4 days after irradiation with 100 r on the ninth day of gestation. ×160.

FIG. 4, *A* and *B*.—Abnormal growths lying in contact with the ectoderm. The great majority of such "cutaneous" tumors were small (*A*), and the occasional one which did reach appreciable size usually showed degenerative changes by the fourth post-irradiation day (*B*). From embryos removed 4 days after irradiation with 100 r on the ninth day of gestation. ×160.

FIG. 5, *A*, *B*, *C*, and *D*.—Clumps of abnormal cells showing varying degrees of protrusion from the outer wall of the developing brain. From embryos removed 2 days after irradiation with 200 r on the ninth day of gestation. ×200.

FIG. 6.—Typical tumors observed on the eighth post-irradiation day. *A:* Large tumor that contains no mitotic figures. *B:* Small tumor, which contains a few mitotic figures, but which is no more advanced in size or differentiation than the tumors seen on the fourth day. *C:* Small atrophic tumor with no mitotic figures. ×160.

FIG. 7.—Dispersal of cells in some of the small tumors. In *A* the cells are still aggregated but are somewhat more loosely arranged than was typically seen. In *B* the cells, although widely scattered, are still recognizable as tumor cells. In *C* few recognizable tumor cells remain, but the area contains much cellular debris and several large multinucleated cells of unknown significance. From embryos removed 3 days after irradiation with 100 r on the ninth day of gestation. ×160.

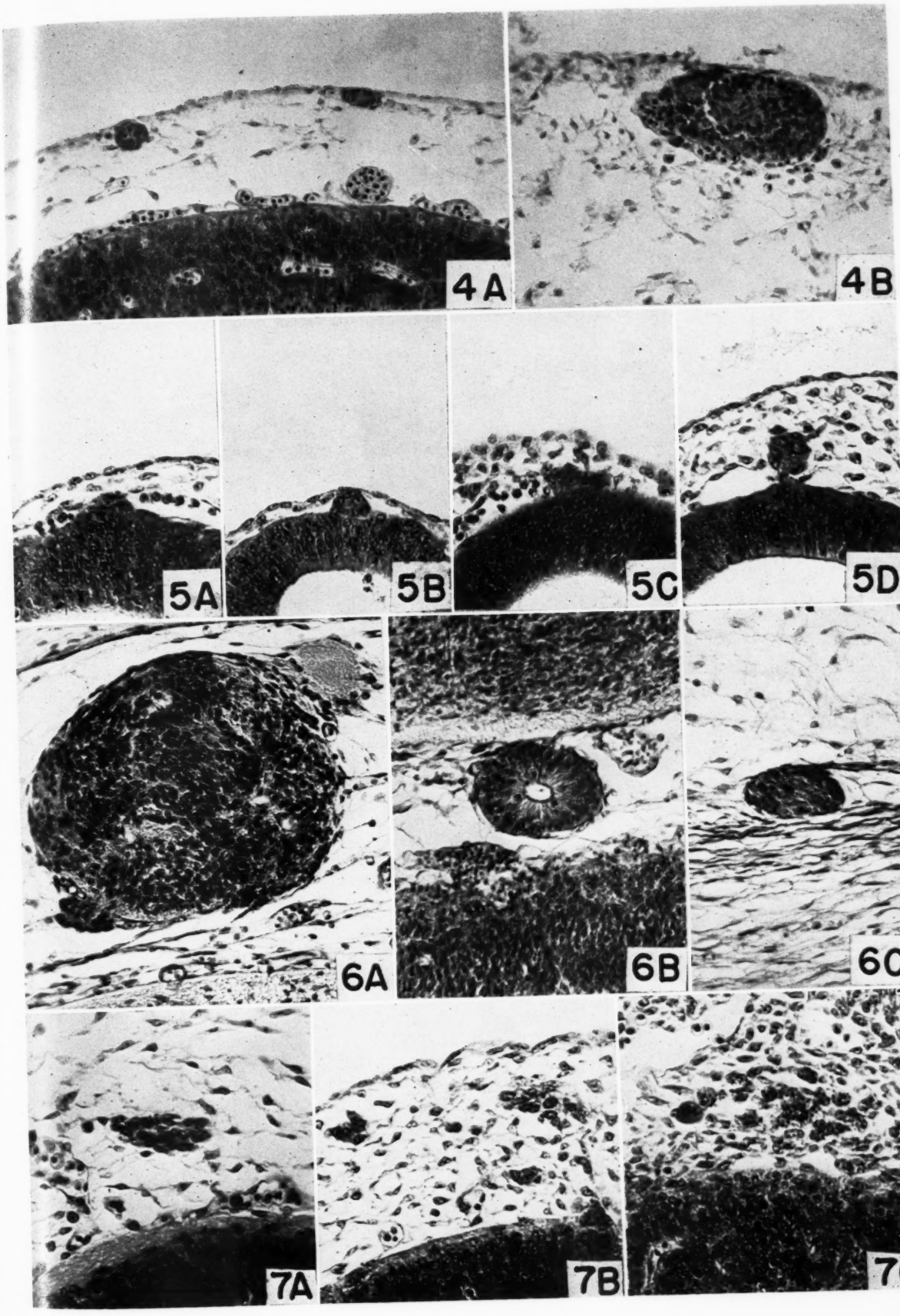

4A
4B
5A
5B
5C
5D
6A
6B
6C
7A
7B
7C

FIG. 8, *A* and *B*.—Atrophic tumors from animals removed 6 days after irradiation with 100 r on the ninth day of gestation. Note the scarcity of blood vessels in the vicinity. ×160.

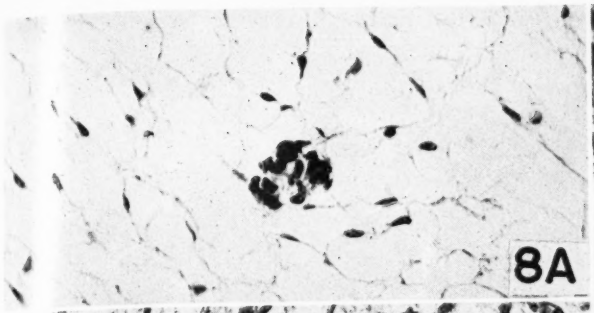
FIG. 9.—Rapidly proliferating tumor from the same animal as that depicted in Figure 8, *B*. ×160.

FIG. 10.—Tumors in newborn rats. *A* is situated between two layers of dura mater in the tentorium cerebelli and is atrophic, despite the fact that a few mitotic figures are present. *B* is tightly wedged between the telencephalon and the diencephalon and appears to be static—that is, neither growing rapidly nor undergoing atrophy. The animals were irradiated with 100 r on the ninth day of gestation. ×160.

FIG. 11.—Large tumor with a peripheral zone containing relatively few cells and numerous filamentous structures, presumed to be nerve processes because of their close histologic resemblance to nerve processes in the brain. From an embryo removed 6 days after irradiation with 100 r on the ninth day of gestation. ×160.

FIG. 12.—Small tumor with a slender chainlike extension of cells out into the mesenchyme. From an embryo removed 6 days after irradiation with 100 r on the ninth day of gestation ×160.

FIG. 13.—Cystic tumor attached to the mesencephalon From an embryo removed 6 days after irradiation with 100 r on the ninth day of gestation. ×160.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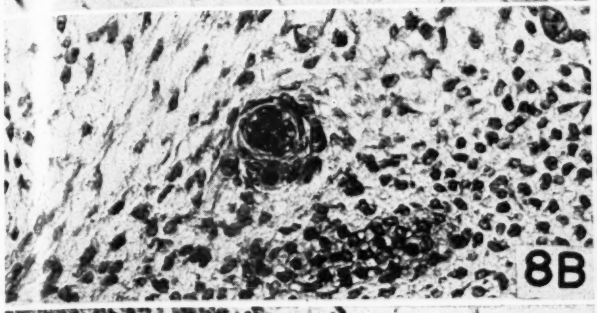
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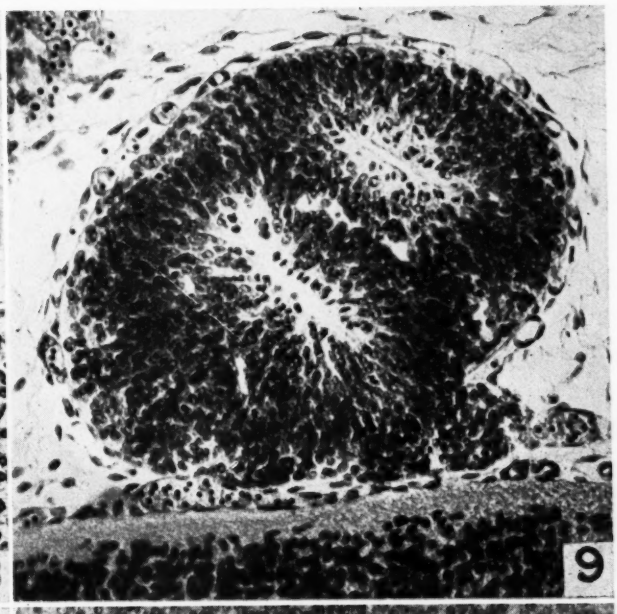
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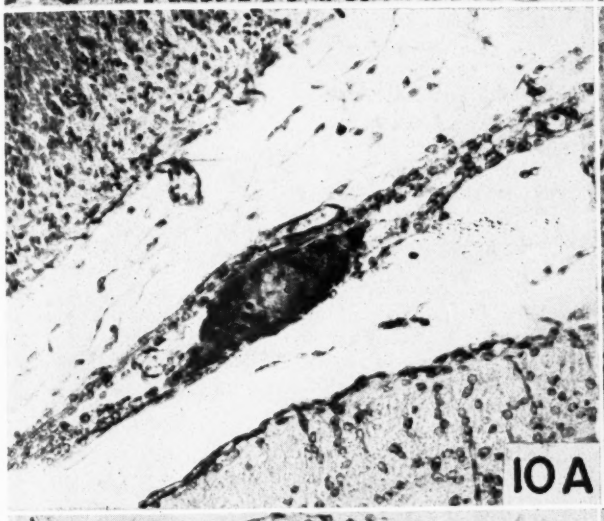
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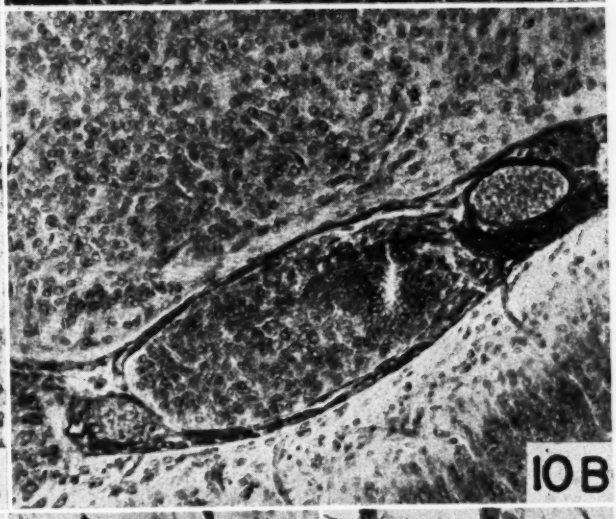
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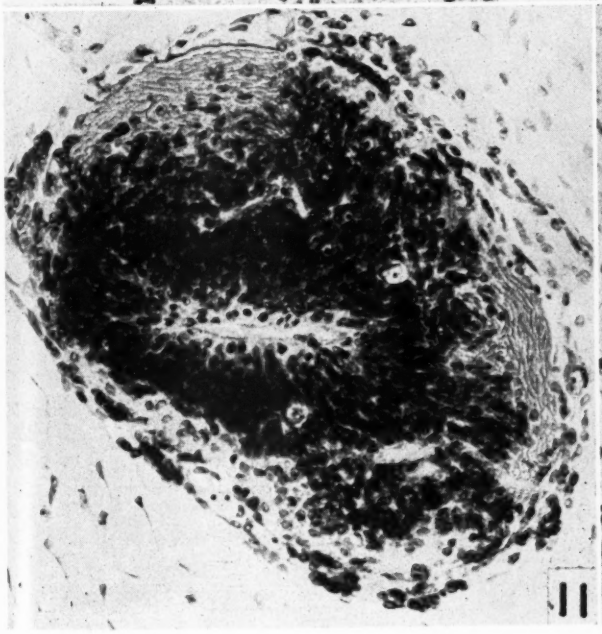
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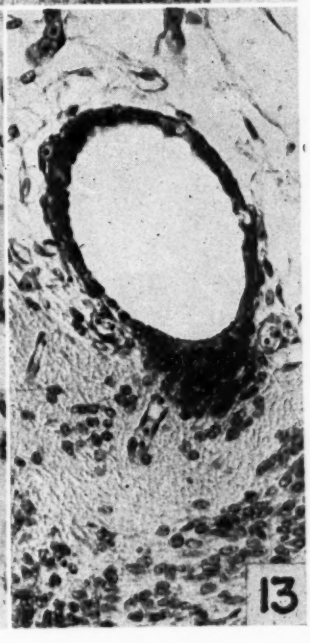
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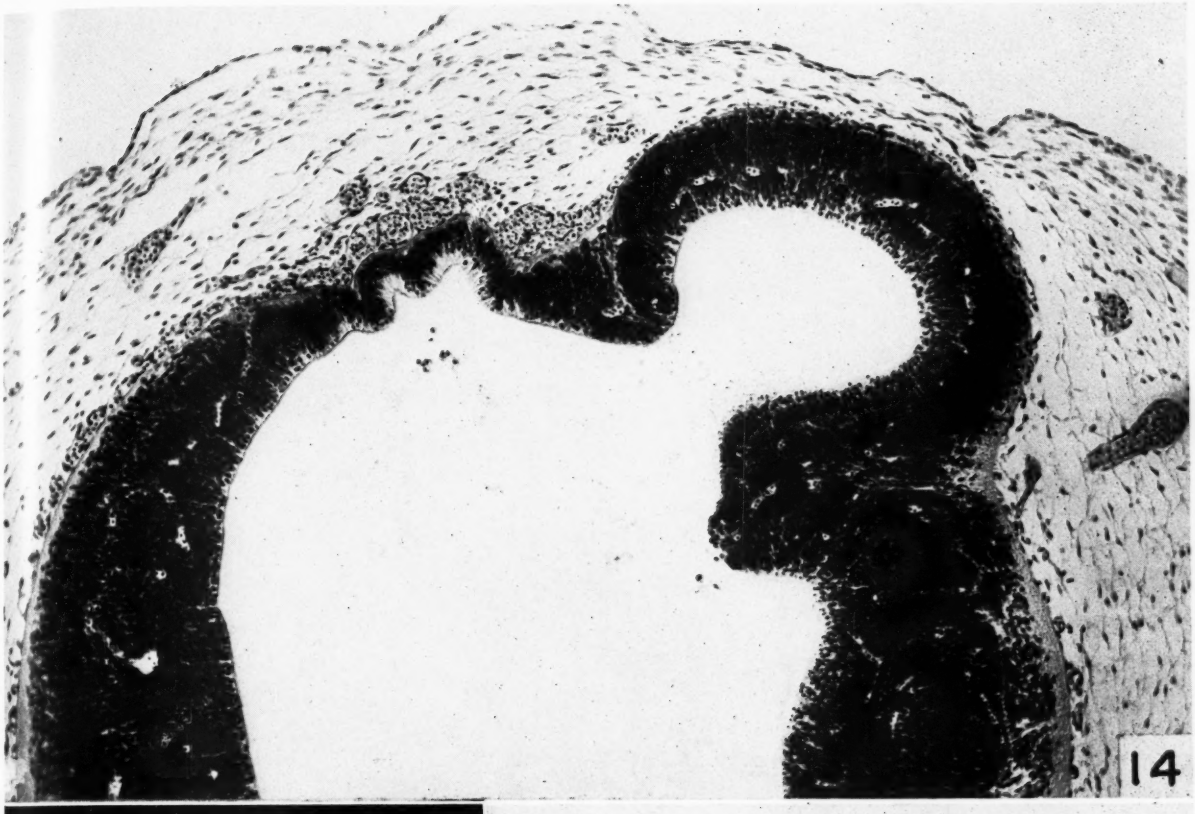
FIG. 14.—Diverticulum on the wall of the diencephalon in an embryo removed 6 days after irradiation on the ninth day of gestation. ×100.

FIG. 15.—Rupture with herniation and eversion of the right telencephalon, in an embryo removed 6 days after irradiation with 100 r on the ninth day of gestation. ×9.

FIG. 16.—Section through the brain of the animal shown in Figure 15. ×20.



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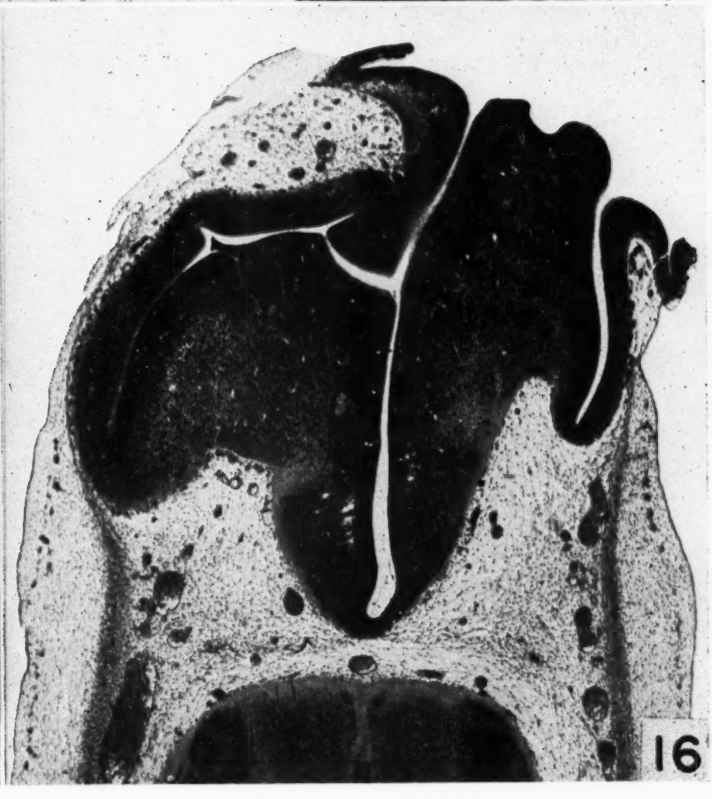

16

Comparative Uptake of Free Amino Acids by Mouse-Ascites Carcinoma Cells and Normal Tissues*

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The growth of a tumor in a wasting animal demands that the normal tissues yield their structural units to the neoplastic tissue. Because such structural elements are constantly released and recombined, the neoplastic cell can gain them from the normal cell merely by seizing upon them with greater avidity, without direct attack upon the normal tissue.

In the investigation reported here, we have considered the behavior of the amino acids, the most prominent of the protoplasmic structural units. The cells of the higher animals (as well as many microbial species) maintain internal environments which are characteristically enriched in the amino acids (7). They do this by taking the various amino acids into the cells against strong concentration gradients. This *concentrative* activity is in some ways as impressive as the similar and related (3) behavior by which the potassium ion is introduced into the cell against a concentration gradient. It is in the enriched cellular medium that protein synthesis occurs. Several observations have led us to propose that the concentrative process for amino acids may play a significant part in the control of growth.

In the pregnant animal, for example, the placenta enriches the fetal circulation in amino acids at the expense of the maternal blood. Not only do the fetal cells live in a richer extracellular medium; they outdo the cells of the adult in further enriching their internal environment (6). This is in spite of their extremely rapid utilization of amino acids for growth. Similarly, the amino acid levels of the liver are sharply elevated during the period of experimental regeneration of this organ (5). The flow of amino acids from the wasting muscles to the growing liver appears to be a result of stepped-up concentrative activity of the liver for amino acids. Several other cases of association between rapid growth and elevated tissue amino acid concentrations have been reported. No one has yet reported the response which is more to be expected—namely, that cellular amino acid levels are dragged down during rapid growth.

According to the above viewpoint, if a tissue were to acquire an increased concentrative activity for amino acids, overgrowth could result. We are presenting the thesis that unusual concentrative powers permit a tumor to capture amino acids from the normal tissues of the host.

As a subject for comparative study of concentrative activity we have used the Ehrlich mouse-ascites carcinoma cell. A mouse inoculated with this tumor may in a week develop cells weighing one-tenth as much as the whole animal did to begin with. These carcinoma cells *in vitro* show concentrative activity so intense as perhaps to explain their competitive success. For example, when we added large amounts of glycine to the cell suspension removed from a mouse, the cellular glycine level rose to a 90-millimolar level with the extracellular at 30 mM—a gradient of 60 mM per liter, enough to cause a considerable osmotic gradient, as indicated by the transfer of water to the cells (3). These gradients surpass any observed *in vitro* with mammalian tissue. The objection may be raised that the uptake of amino acids should be compared in the whole mouse where the competition actually occurs. This has been done with the results recorded below.

EXPERIMENTAL

Male mice of strain A (Jackson Memorial Laboratory, Bar Harbor, Maine) were inoculated with 0.2 ml. of ascitic fluid from a mouse having a well developed carcinoma-cell ascites. Two to 4 days later the animal was fasted 3 hours, and then fed the glycine or L-alanine in two doses, the first one 25 mM/kg of body weight, and the second one an hour later, 15 mM/kg. The animals were sacrificed for analysis 1 hour after the last dose. The purpose of this dosage schedule was to maintain a nearly maximal rate of absorption of the amino

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acid over nearly 2 hours. Analysis of the gut contents at the end of the experiments showed the presence of 16–26 per cent of the glycine fed. When 40 mM of alanine was fed according to the same schedule, only 2.3 per cent of the alanine remained unabsorbed. Therefore, the dosage of alanine was increased to 60 mM/kg, in two doses of 40 and 20 mM. In this case 17 per cent of the alanine was recovered in the alimentary canal.

The animals were killed by decapitation. In most cases blood was collected in a heparinized tube. The ascitic fluid was collected rapidly after opening the abdomen. The liver and the musculature of the hind legs were removed quickly, weighed on a direct-reading torsion balance, and ground in a mortar with sand in the presence of 10 parts of saturated aqueous picric acid. The tumor cells were separated by centrifugation in a tared tube at 38° C., then weighed and extracted with saturated picric acid. The blood plasma and the cell-free ascitic fluid were treated with 5 volumes of aqueous picric acid. The extracts were analyzed for glycine or alanine by the methods of Alexander, Landwehr, and Seligman (1, 4) and of Alexander and Seligman (2).

RESULTS AND DISCUSSION

Chart 1 shows the powerful uptake of glycine by the carcinoma cells *in vitro* (cf. 3). The line drawn on this chart represents a 50-mM gradient,

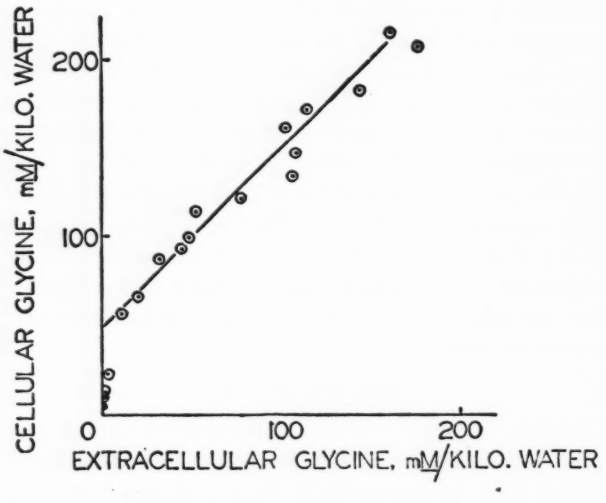

CHART 1.—Relation between extra- and intracellular glycine. Line represents 50 mM gradient.

that is, the cell water richer than the extracellular fluid by 50 mM of glycine/kg. It should be emphasized that the gradient does not remain constant at low concentrations; actually, the curve slopes off toward the origin. The indications are that at zero extracellular glycine concentration there will be no free glycine in the cells.

The results obtained with the intact tumor-bearing mouse are shown in Table 1 (for glycine) and Table 2 (for alanine). Because the content of extracellular water for the various tissues was not determined, the results have been expressed in mM/kg of fresh tissue, rather than in mM/kg of cellular water. With feeding of glycine the plasma glycine level was brought up to 12–19 mM, compared to the normal values of about 0.4 mM. In the ascitic fluid the levels were not quite so high—8–14 mM. The lower values here may have been due to limitations in the rate of diffusion between the two fluid compartments relative to the rate of removal of glycine from the fluid by the cancer cells. In the unfed animal the differences are smaller (3). In any case, the three types of cells were concentrating amino acid from fluids of similar concentration, the cancer cells being slightly handicapped in the acquisition of glycine by the lower levels in the fluid bathing them.

It may be seen that the neoplastic cells reached the highest glycine levels of the three tissues, the order being carcinoma cells > liver > muscle. If approximations are made for the cellular and extracellular water in order to calculate the distribution ratios (glycine in cellular water)/(glycine in extracellular water), the concentrating activity of the tumor cells is found to be clearly and consistently greater than that of the liver cells and much

TABLE 1

DISTRIBUTION OF FREE GLYCINE AFTER FEEDING TO MICE BEARING THE ASCITES TUMOR
(Values are in mM/kg of tissue)

Condition of mouse	Blood plasma	Liver	Muscle	Ascitic plasma	Carcinoma cells
Fasted	0.4*	1.8(±0.3)*	2.5(±0.2)*	0.3*	3.9*
Glycine-fed		20	6.1		34
" "	11.9	33	6.6	8.3	42
" "	17.5	51	13.9	12.0	100
" "	18.9	44	14.1	14.2	52

* These averages were obtained with tumor-bearing mice of the same strain. The values in parentheses are standard deviations (four observations).

TABLE 2

DISTRIBUTION OF FREE L(+)ALANINE AFTER FEEDING TO MICE BEARING THE ASCITES TUMOR
(Values are in mM/kg of tissue)

Condition of mouse	Blood plasma	Liver	Muscle	Ascitic plasma	Carcinoma cells
Fasted	0.8*	5.5(±1.8)*	3.6(±0.6)*	0.76*	5.5*
Alanine-fed	1.20	7.8	5.5	2.3	15.5
" "	7.7	19.0	9.0	7.2	16.9

* Averages obtained with ascites tumor-bearing mice of the same strain. The values in parentheses are standard deviations (three observations).

greater than that of the muscle cells. In the experiments with alanine feeding, the three tissues fall in the same order as to alanine uptake in one case; in the other, the liver surpasses the neoplastic cell with regard to alanine accumulation.

There can be no question that the net synthesis of protein had been proceeding much faster in the multiplying neoplastic cells than in the other tissues of these mice. Were isotopically labeled alanine administered, undoubtedly the more rapid incorporation of the amino acid into protein in the neoplastic cell than in other cells could be demonstrated, as was done by Zamecnik, Frantz, Loftfield, and Stephenson with rat hepatomas (8). The present experiments have a quite different purpose. They show that the neoplastic cells have a greater ability to accumulate free amino acids than other cells; this should give the protein-synthetic mechanisms in the cancer cells an advantage, which is perhaps expressed in their rapid growth and in the flow of amino acids from normal tissues to tumor in advanced neoplasia.

The liver occupies a special position in amino acid metabolism, not only as a protein-synthesizing organ, but also in its capacity of destroying amino acids by deamination and by other reactions. It is not maintained that the greater accumulation of added amino acids by the liver than by the muscle, or by almost any other tissue, implies that the liver ought to grow faster than muscle or other tissues. Certain concentrative activities are characteristic of each organ for each amino acid. Growth has been shown in several instances to be associated with increases of these characteristic concentrative activities. Unfortunately, the concentrative activities of epithelial cells which are homologous to the carcinoma cell are not yet subject to measurement. We do regard as significant the finding that the carcinoma cells exceed even the highly active liver cells with regard to the accumulation of free amino acids.

SUMMARY

When mice bearing the Ehrlich ascites tumor were fed glycine or L-alanine, the carcinoma cells were more active in the accumulation of the free amino acid than the cells of liver or muscle. This superiority in amino acid accumulation is considered a significant factor in the growth and multiplication of the neoplastic cell in a wasting animal.

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Inhibition of the Pasteur Effect in Yeast by Tumor Extracts and Differences in Lability of Sulfur in Normal and Tumor Tissues*

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Some time ago we reported (12) the preparation of extracts from bovine semen which stimulated the respiration and anaerobic glycolysis of epididymal spermatozoa of the same species. The extracts inhibited the Pasteur effect completely in baker's yeast and to a significant extent in bovine epididymal spermatozoa. The biological activity of the extracts seemed to result, at least in part, from their ability to prevent energy utilization by uncoupling oxidative phosphorylation (8, 11). More recently, it has been possible to separate the yeast fermentation stimulating activity from that which causes uncoupling of oxidative phosphorylation in rat kidney or liver preparations.

In freshly ejaculated bull semen the yeast-stimulating substance appeared to be liberated slowly from the sperm cells in a more or less inactive form (water-soluble) under the influence of certain enzyme systems present in seminal fluid; but it could readily be released in the active state from spermatozoa or whole semen by mild alkaline hydrolysis and then could be extracted with nonpolar solvents. Similar yeast-active extracts were also obtained from testicular tissue. The yeast-active principle has recently been isolated in crystalline form from hog testis extract and has been identified as elemental sulfur. The sulfur precursor in semen, as elaborated from the sperm cells, appeared to be a nondialyzable polypeptide, other than glutathione, from which elemental sulfur might be split off by enzymes of the thionase type. These have been found present in both seminal fluid and yeast. The details of this extensive series of investigation will be presented elsewhere.[1]

* Supported by a grant from the American Cancer Society, on recommendation by the Committee on Growth of the National Research Council, and by the Brittingham Trust Fund. A preliminary report was presented before the American Association for Cancer Research (Cancer Research, 11:251, 1951).

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Before the active principle was identified, a variety of animal and human tumors was analyzed for the active substance on the speculation that such regulatory substances might be associated with the processes of cell division and growth. Surprisingly enough, extracts from all the samples of tumors analyzed showed a remarkably high degree of activity, whereas those from normal tissues except testis showed negligible activity in the yeast fermentation test system. In view of the well emphasized role of sulfhydryl groups in the phenomena of cell division and experimental carcinogenesis (1, pp. 170–79, 182–84), this striking difference between normal and tumor tissues in the lability of sulfur was studied further.

MATERIALS AND METHODS

Preparation of tissue extracts and method of assay.—Fresh or frozen tissue was homogenized with cold acetone in a Waring Blendor, and the acetone powder was collected by filtration. The powder was rapidly dried in a vacuum desiccator and stored, if necessary, at −5° C. One gm. of acetone powder was suspended in 12 ml. of 0.17 M NaOH and hydrolyzed in a boiling water bath for 10 minutes with frequent stirring. The solution was quickly cooled to room temperature and acidified to about pH 3 with 1 N HCl. The material was then transferred to centrifuge tubes and shaken vigorously with equal volumes of carbon tetrachloride (reagent A.C.S.) for 2 minutes. The carbon tetrachloride layer was separated by centrifuging, and the process of extraction was repeated 3 times. The combined carbon tetrachloride extracts were evaporated to 1 or 2 ml. at room temperature under reduced pressure. Aliquots were pipetted directly into dry Warburg flasks, the solvent evaporated off at room temperature under suction, and assayed manometrically by the yeast fermentation system consisting of 1.5 ml. of fermentation medium (glucose, 7.92 gm.; sodium acetate, 2.05 gm.; acetic acid, 1.5 gm.; KH_2PO_4, 1.36 gm.; $MgSO_4 \cdot 7H_2O$, 0.324 gm.; $MnSO_4 \cdot H_2O$,

0.102 gm.; vitamin B_1, 0.1 gm.; niacin, 0.1 gm.; vitamin B_6, 50 mg.; calcium-pantothenate, 50 mg.; and water to make 500 ml.), 1 ml. of a 0.5 per cent suspension of fresh Red Star yeast cake (dry wt., approx. 1.5 mg.) and 0.5 ml. of water. Fermentation was studied at 30° C. for 1 hour after an equilibration period of 5 minutes. Rat tumors used in this study were excised within 2 weeks after being transplanted and were separated from necrotic tissue as completely as possible. Human tumor samples were carefully separated from adipose tissues, whenever necessary.

Total sulfur in tissue acetone powders was determined by an adaptation of the method described by Klimenko (10), after combustion in a Parr bomb. Sulfate was precipitated with a suspension of barium chromate, and liberated chromic acid was titrated iodometrically.

RESULTS AND DISCUSSION

Inhibition of Pasteur effect in yeast by elemental sulfur.—The effect of elemental sulfur in inhibition of Pasteur effect in yeast is shown in Chart 1.

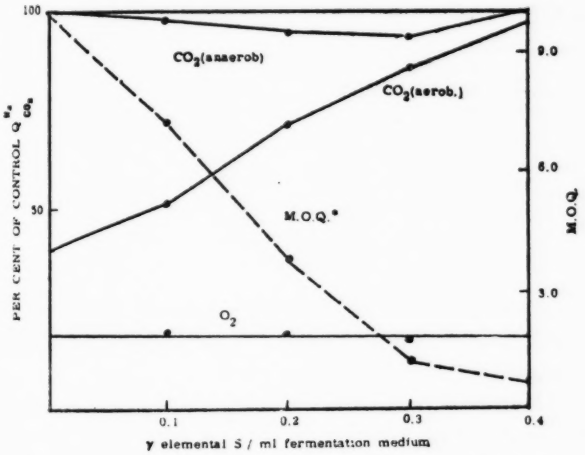

CHART 1.—Inhibition of Pasteur effect in yeast by elemental sulfur.

Results are expressed as per cent of $Q^{N_2}_{CO_2}$ in control. Aerobic CO_2 production was corrected for respiratory CO_2, assuming R.Q. = 1. The control rates were: $Q^{N_2}_{CO_2} = 301$, $Q^{air}_{CO_2} = 120$, and $Q_{O_2} = -55$.

* M.O.Q. (Meyerhof Oxidation Quotient) $= \dfrac{Q^{N_2}_{CO_2} - Q^{air}_{CO_2}}{Q_{O_2}/3}$.

The metabolic rates are expressed as the percentage of the rate of anaerobic fermentation in the control. It will be seen that, with increasing concentration of sulfur, aerobic fermentation gradually increased up to the anaerobic level, while respiration and anaerobic fermentation remained practically constant. The Pasteur effect was expressed in terms of the "Meyerhof Oxidation Quotient," which is:

$$\frac{(Q^{N_2}_{CO_2} - Q^{air}_{CO_2})}{Q_{O_2}/3}.$$

This quotient describes the efficiency with which oxidative processes prevent fermentation of carbohydrate (3). From a value of 10 in the control, the M. O. Q. progressively declined with increasing concentration of sulfur until at 0.4 μg of sulfur/ml of fermentation system it practically came down to zero. In other words, the Pasteur effect was completely abolished at this level of sulfur. However, it should be pointed out that H_2S, produced from elemental sulfur by yeast, is probably responsible for the effect on fermentation. In measuring yeast respiration, KOH is used in the center well, and this agent may absorb H_2S as rapidly as formed, thus preventing it from inhibiting oxygen consumption. In the aerobic fermentation test system no KOH is present, and the H_2S formed may exert its full effect on the cytochrome system. The amount of sulfur which gives a maximum response (0.4γ/ml) could yield 1.3×10^{-5} M H_2S. Whether this would be a sufficient concentration to inhibit yeast respiration completely seems doubtful.

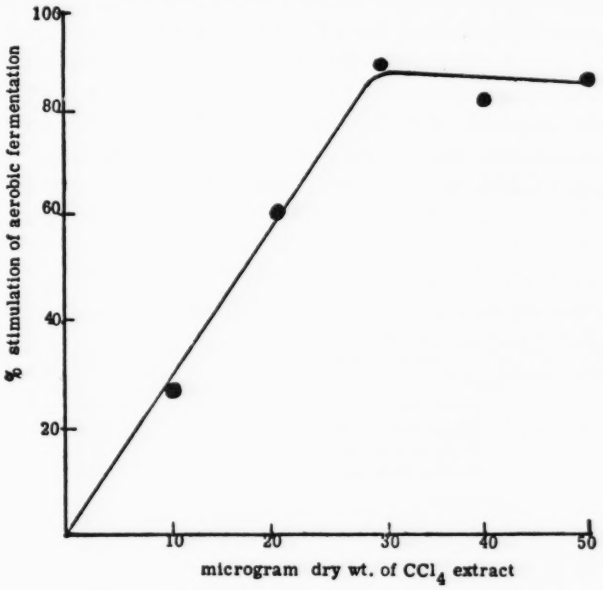

CHART 2.—Effect of CCl_4 extract from human breast carcinoma (72830) acetone powder on aerobic fermentation of yeast.

Effect of tissue extracts on aerobic fermentation of yeast.—The typical dose response curve with active tumor extracts is shown in Chart 2, in which the per cent stimulation of fermentation has been plotted against μg. dry matter in carbon tetrachloride extract from a sample of human

breast carcinoma. As little as 30 μg. of dry matter per flask brought about a maximum stimulation of aerobic fermentation. There was no effect on respiration or anaerobic fermentation at these levels. The results of representative experiments with different types of tumor tissues are summarized in Table 1. The figures in the first column are the control rates of fermentation in the different experiments. The maximum response in each case is italicized. In all cases maximum stimulation, amounting to 80–100 per cent over the control, was obtained with extracts equivalent to 20–50 mg. of acetone powder. These levels corresponded to dry matters ranging from 5 to 15 μg/ml of fermentation mixture. It may be pointed out here that out of five different samples of stomach carcinoma analyzed, one particular sample (No. 72656) showed very low activity. From cytological observations[2] this specimen was reported to be mostly fibrous tissue containing not more than 10 per cent of tumor cells. This fact was not known to the authors at the time the sample was assayed.

TABLE 1

EFFECT OF CCl_4 EXTRACT OF ALKALI-HYDROLYZED TUMOR TISSUE ACETONE POWDER ON AEROBIC FERMENTATION OF YEAST

Extract equiv. to mg. acetone powder	Q_{CO_2} 0	10	20	30	40	50
Rat:						
Hepatoma	171	197	286	307	*314*	309
Flexner-Jobling carcinoma	200	236	*338*	334	328	336
Walker 256 carcinosarcoma	195	344	*355*	354	354	336
Jensen sarcoma	206	320	340	*356*		
Human:						
Breast carcinoma No. 72830	193	245	312	*368*	353	362
Sigmoid " " 72576	187	203	307	333	*344*	335
Stomach " " 72578	155	200	284		292	*298*
" " " 72751	183	191	255	292	306	*317*
" " " 72852	173	199	285	*302*	300	292
" " " 72854	180	198	238	295	306	*322*
" " " 72656*	190	193		178	178	184
Melanoma No. 72496	181	313	*338*	327	324	336

* See note in text concerning this specimen.

Data from parallel experiments with extracts from a variety of normal tissues including certain embryonic tissues are presented in Table 2. With the exceptions of bull semen and hog testis, extracts from the normal adult tissues tested were practically inactive; extracts from rat embryo and fetal calf liver showed activity only at very high levels, although fetal calf kidney did not show any activity at all. In the case of bull semen, extract from as little as 5 mg. of acetone powder, corresponding to less than 2 μg. of dry matter/ml of fermentation mixture, was sufficient to give a maximum response. The presence of free sulfur in all the active extracts was confirmed by spot test (7), after the active principle from testis extract had been identified as elemental sulfur.

Total sulfur in acetone powders of normal and tumor tissue.—Analysis of the acetone powders of normal and tumor tissues used in this study did not reveal any marked difference in their total sulfur content. The results are shown in Table 3. Rat tumors appeared to be somewhat richer in total sulfur content compared to rat muscle, but the values for rat liver tumor and normal liver were practically identical. The total sulfur in acetone powders of bull semen and hog testis was fairly high. In spite of the relatively high sulfur content of acetone powders of rabbit muscle extract and calf thymus, these specimens did not give active extracts.

Data reported in the literature on the total sulfur content of neoplastic tissues and their normal counterparts are very few. The total sulfur content of rat sarcoma, mouse carcinoma, and Rous sarcoma was found by Toyoda *et al.* (16) to be no greater than that of normal kidney or spleen, though much larger than that of skeletal muscle, blood, heart, or liver. Values for various organs of sarcoma-bearing rats were found to be generally higher than those from normal rats (16). Brown and Kauder reported (2) that the sulfur content of rabbit skin decreased with decreasing rate of growth from birth to maturity and that rapidly growing mouse sarcoma contained more sulfur than slowly growing tumors. On a dry weight basis, the total sulfur of rat hepatoma has been reported to be slightly higher than in normal liver: 1.15 per cent and 0.92 per cent, respectively (9). Although available evidence indicates a slightly higher sulfur content in tumor tissues as compared

[2] We wish to thank Dr. J. W. Harman, Department of Pathology, for the supply of human tumor samples and histological information on the samples.

to normal tissues, common analytical procedures would not detect the mild alkali-labile fraction, since the free sulfur obtained in the CCl_4 extract of the hydrolysate represents a very small fraction of the total sulfur of the tumor samples.

Under the conditions of hydrolysis and extraction specified above, elemental sulfur could be obtained in large amounts from sulfur-rich proteins such as keratin (chicken feathers) and albumin (crystalline egg and bovine serum albumin), but the free amino acids, methionine, cystine, or cysteine did not yield elemental sulfur. (Cysteine and cystine are known to be stable to mild alkali treatment [4]. Evidence has been presented, however, to indicate that cystine derivatives, in which the carboxyl group of cystine is in peptide or analogous combination, are more sensitive to desulfurization by alkali than is cystine itself [13].) Glutathione yielded approximately 0.2 per cent of its sulfur to the CCl_4 extract when treated as described; this was much too small to account for the activities in tumor extracts. Furthermore, total glutathione content of tumor tissues is generally believed to be lower than that of normal tissues, although there is some confusion as regards the concentrations of total sulfhydryl compounds in tumors (15, p. 114). An explanation of the high lability of sulfur in tumor acetone powders in terms of an excessive quantity of albumin in tumor tissues can, perhaps, be ruled out on the basis of the diminished content of albumin in tumors as compared to normal tissues (15, p. 101). There is a high concentration of sulfur-containing amino acids in bull spermatozoa, and the presence of keratin-like protein in the membrane of the sperm head was indicated (17). The high sulfur content in bone marrow (6) and testicular tissue, where specialized cell formation is constantly going on, also seems to indicate the strategic involvement of sulfur-rich proteins in the phenomena of cell division. A recent investigation has demonstrated that white cells of leukemic patients contain larger amounts of -SH containing substances (determined as glutathione) than the white cells of normal persons (5).

Lability of sulfur in acetone powders of particu-

TABLE 2

EFFECT OF CCl_4 EXTRACT OF ALKALI-HYDROLYZED TISSUE ACETONE POWDER ON AEROBIC FERMENTATION OF YEAST

Extract equiv. to mg. acetone powder	Q_{CO_2}									
	Rat liver	Rat muscle	Rat spleen	Rabbit muscle	Beef brain	Calf kidney (fetal)*	Calf liver (fetal)*	Rat embryo†	Hog testis	Bull semen
none	188	214	190	190	170	171	187	165	166	150
2										235
5										*303*
10	186	214	192	187		180	185	183	200	302
20	181	218	206		193	167	189	175		280‡
30		212					197		270	
40	190	218	206	191	158		210	195		
50		204				185	206		*307*	
80	191				183			(284)		
100	199		190	196	179	191	295		292‡	
200	(231)		204	198	180		*330*			

Figures in parentheses represent significant activity, but higher levels were not tested.
* At approximately eighth month of gestation.
† At approximately eighth day of gestation.
‡ Respiration partially inhibited at this level.

TABLE 3

TOTAL SULFUR IN ACETONE POWDERS OF NORMAL AND TUMOR TISSUES

Tissue	Total S (per cent)	Tissue	Total S (per cent)
Rat muscle	1.12	Human:	
Rat liver	1.32	Stomach carcinoma No. 72578	0.97
Rabbit muscle extract*	1.57	" " " 72656	0.78
Calf thymus	1.68	" " " 72751	1.18
Beef brain	1.26	" " " 72852	1.08
Hog testis	1.61	" " " 72854	1.24
Bull semen	1.95	Rat:	
		Walker 256 carcinosarcoma	1.55
		Flexner-Jobling carcinoma	1.55
		Primary hepatoma	1.34

* Water-soluble fraction of ground rabbit muscle.

late fractions of normal rat liver and Walker carcinoma.—In view of the high lability of sulfur in tumor proteins towards mild alkaline hydrolysis, it seemed of interest to separate the particulate fractions in tumor cells and assay extracts from acetone powders of individual cellular components by yeast fermentation.

To determine whether the alkali-labile sulfur was derived from any particular cell component, fractionations of freshly excised Walker carcinoma were carried out by the method of Schneider (14), with the use of 8.5 per cent sucrose. After separation, each of the sedimentable fractions—nuclei, mitochondria, and microsomes—was washed once with sucrose and twice with isotonic KCl before making the acetone powders. The supernatant fraction in sucrose was precipitated with 4 vol. of acetone, the precipitate redissolved in isotonic KCl and then reprecipitated with acetone. Carbon tetrachloride extracts were made from hydrolysates of these acetone powders and assayed for activity in the usual manner by the yeast fermentation system. The activities were compared to those of the corresponding fractions of normal rat liver prepared exactly in the above manner. Rat liver was selected as representative of normal tissue, since the sucrose fractionation procedure gives clean separations of its components. The results are presented in Table 4. The activities are expressed as units/100 mg of acetone powder. One unit of activity was defined as that amount which gave a 50 per cent stimulation of yeast fermentation over the control aerobic rate.[1] It is interesting that the activity of the extracts of each of the different cellular fractions of tumor was significantly higher than that of the corresponding fractions of normal tissue. Much more sulfur seemed to be liberated from the acetone powders of the liver cellular components than from powders of the whole tissue (cf. Table 2). There appeared to be an absolute difference in the sulfur liberated by mild alkali from the nuclear fraction of normal and tumor tissues. This strongly suggests a structural difference in the sulfur-containing protein of the respective nuclei. A quantitative study of the -SH and -S-S- groups in the nuclear proteins in tumor tissues might lead to useful information, especially in view of the once accepted idea that the primary reaction of a carcinogenic hydrocarbon consisted in the opening of a proteinoid -SS- linkage with the attachment of the carcinogen to one sulfur atom (1, p. 182).

TABLE 4

ACTIVITY OF CCl_4 EXTRACTS FROM ALKALI-HYDROLYZED ACETONE POWDERS OF PARTICULATE FRACTIONS OF NORMAL RAT LIVER AND WALKER CARCINOSARCOMA ON YEAST FERMENTATION TEST SYSTEM

	Units*/100 mg of acetone powder			
Tissue	Nuclei	Mitochondria	Microsomes	Supernatant
Normal liver	0.0†	3.2	1.66	4.0
Walker carcinosarcoma	1.6	12.5	15.4	11.5

* One unit is arbitrarily defined as the quantity of extract giving a response of 50 per cent stimulation of aerobic fermentation of yeast over the control.

† Extracts equivalent to as much as 160 mg. of acetone powder were tested.

SUMMARY

1. Carbon tetrachloride extracts from mild alkali-treated acetone powders of a variety of rat and human tumors inhibited the Pasteur effect in baker's yeast. Complete inhibition was obtained with extracts containing dry matter ranging from 5 to 15 μg/ml of yeast fermentation assay system. Similar extracts from normal tissues, except testis and semen, were inactive. Embryonic tissue extracts showed activity only at high levels.

2. After the active principle, which was originally discovered in mammalian spermatozoa (12), had been identified as elemental sulfur,[1] the acetone powders of normal and malignant tissues were analyzed for total sulfur. Total sulfur content of rat tumors (Flexner-Jobling, Walker 256, and primary hepatoma) were slightly higher than that of rat muscle; the values for normal liver and hepatoma were, however, identical.

3. In view of the greater lability of sulfur in tumor, a structural difference between the sulfur-containing proteins of normal and tumor tissues is postulated. This difference was relative in mitochondrial, microsomal, and supernatant fractions and absolute in the nuclear fractions of the respective tissues.

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Failure To Observe Pentolysis by the Serum of Rats Bearing Malignant Tumors*

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In 1944, Bevilotti (1) reported that the red corpuscles of patients with malignant tumors degrade pentoses *in vitro*, while the red cells of normal subjects, or of patients with benign tumors, do not have this effect. Menkes (5–8) observed a similar action by blood serum. He found that when the sera of patients with malignant tumors were incubated at 37° C. with pentoses (D-ribose, D-xylose, D-arabinose, or L-xylose) degradation of the pentose occurred with formation of lactic and pyruvic acids. Normal sera and the sera of patients with benign tumors and chronic diseases, such as tuberculosis, cirrhosis, diabetes, and nephritis, did not exhibit this effect. Menkes called this process "pentolysis," since it appeared to be a degradation somewhat similar to glycolysis of hexoses. Menkes considered a degradation of pentose greater than 10 per cent in a 2-hour incubation period a positive diagnosis of cancer. He found an unavoidable error of 2 per cent, and pentolysis values between 5 and 9 per cent were considered doubtful.

Steen (10, 11) carried out studies on the serum of rats in which tumors had been developed by injecting 20-methylcholanthrene. Positive pentolysis tests were obtained in 20 of 22 blood samples from rats bearing experimentally induced tumors. This work supported Menkes' contention that pentolysis produced by blood serum is a manifestation of carcinogenesis.

Kubowitz and Wieding (3) were unable to confirm the postulation of Menkes in experiments with rats bearing the Walker-Karzinen tumor.

The data reported in this paper are the results of a study designed to examine the validity of the so-called pentolysis test of blood serum for the presence of malignancy. During the course of the work, a critical evaluation of analytical methods for the determination of pentoses and furfural was made. Tests were carried out upon the sera of eighteen rats with tumors induced by injecting 20-methylcholanthrene and of ten rats with transplanted tumors.

After this paper had been prepared for publication, a report by Steen (12) appeared in which the author stated "the results of these determinations on a large scale, as well as those of an earlier paper, are thus seen to fall within the limits of the experimental errors of the methods employed for determining the amount of free pentoses. Accordingly, the so-called phenomenon of pentolysis in the blood of rats bearing induced sarcomas is a misnomer."

EXPERIMENTAL

Wistar strain rats, obtained from Carworth Farms, were injected with a single subcutaneous dose of 20-methylcholanthrene (3 mg. in 0.5 ml. of mineral oil) in the abdominal wall. Control animals were injected similarly with mineral oil only. All blood samples were drawn by intracardiac puncture. The blood was allowed to clot, and the serum was removed after centrifugation within 30 minutes. The tests were carried out immediately thereafter. The tumors were removed, fixed in formaldehyde solution, and later examined histologically.

The incubation procedure was identical with that proposed by Menkes (5–8), except that sterile conditions were not maintained. Since the tests for pentose degradation were negative at first and continued to be negative throughout the study, we saw no reason to attempt to eliminate from the digestion mixture micro-organisms that might destroy pentose. Three methods for the determination of pentose were used. These were the procedures of Brachet (2), Roe and Rice (9), and Mejbaum (4). With each of the three methods, pentose was determined in unincubated samples

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(one tube containing serum and water, the other tube containing serum and ribose) and in samples incubated for 2 hours at 37° C. (serum and water in one tube and serum and ribose in the other). Color was measured by the Evelyn colorimeter, with the use of unincubated and incubated samples of serum and water as control blanks for the unincubated and incubated samples containing serum and ribose. Pentolysis was calculated by dividing the difference in the densities of the unincubated sample and the incubated sample by the density of the unincubated sample; this value times 100 equals the per cent change in pentose.

Menkes (5–8) determined pentose in incubated samples of serum and water and serum and ribose. Colorimetric measurements were made with a Hilger-Spekker colorimeter, and the amount of pentose was calculated from a standard curve. The value of the blank (serum and water) was subtracted from the value of the sample of serum and ribose, and the figure thus obtained was referred to the amount of pentose originally placed in the tube; this gave the per cent of pentolysis. Our method of calculation more accurately estimates change in ribose content, because it is a direct comparison of two identical samples, one incubated and one not incubated.

RESULTS

In Table 1 are shown the results of pentolysis tests performed on eighteen rats with tumors induced by injection of 20-methylcholanthrene. A pentolysis value as great as 10 per cent was not observed in the serum of any of these tumor-bearing rats, and only two sera showed an apparent degradation of pentose greater than 5 per cent. These data, therefore, do not show any evidence of a pentose-destroying factor in the serum of rats bearing tumors produced with 20-methylcholanthrene.

Further studies were made upon rats bearing transplanted tumors with the same incubation procedure and the Roe and Rice (9) and Mejbaum (4) methods for the determination of pentose. Results are shown in Table 2. The decrease in pentose in all instances was less than 5 per cent. These results are further evidence for the nonexistence of a pentose-destroying factor in the serum of animals bearing malignant tumors.

DISCUSSION

Since proof of the existence of a pentose-degrading factor in serum is dependent upon the accuracy of the method used for the determination of pentose, three different analytical methods were

TABLE 1

RESULTS OF PENTOLYSIS TESTS UPON SERUM FROM RATS BEARING TUMORS INDUCED BY INJECTION OF 20-METHYLCHOLANTHRENE

Rat no.	Days after injection	Pathologic diagnosis	Per cent change in pentose — method of analysis: Brachet	Roe-Rice	Mejbaum
3		Control	+29.4		
8		"	+ 7.6		
9		"	+ 2.9		
10		"	− 1.2		
29		"		0.0	
30		"		−2.9	−3.6
1	140	Cyst	− 3.9		
2	141	"	+ 3.3		
4	145	"	− 3.9		
5	146	"	− 3.6		
6	147	Fibrosarcoma	− 0.2		
11	154	Cyst	+ 9.4		
12	155	Fibrosarcoma	+11.9		
13	158	"	+ 9.8		
14	159	"	+ 8.7		
15	161	"	+ 5.6		
16	165	"		−3.1	−5.9
17	166	Carcinoma		−1.7	+0.1
18	167	"		−4.2	−4.1
19	168	Chronic inflammation		+2.1	−3.9
20	169	Fibrosarcoma		+0.3	
22	173	"		+0.6	−3.3
23	174	"		−1.0	−4.6
24	175	"		−0.2	−0.1
25	176	"		−4.2	−4.0
26	181	Cyst		−5.3	−1.4
27	182	Fibrosarcoma		−0.4	−4.3
28	183	"		−3.3	−3.3
31	188	Cyst		−0.3	−1.6
32	189	Fibrosarcoma		−3.7	−5.5
33	190	"		−3.4	−2.9
35	194	"		−1.7	−2.6

used, and special attention was directed to a study of the analytical principles involved. Table 3 shows a comparison of the precision obtained by the three methods used. Average variation of the duplicate determinations for both the unincubated and the incubated samples show that the greatest precision in the determination of pentose was obtained by the Roe and Rice method. Results by the Mejbaum method were next in order of precision, and values by the method of Brachet had the least

TABLE 2

RESULTS OF PENTOLYSIS TESTS UPON SERUM FROM RATS BEARING TRANSPLANTED TUMORS

TYPE OF TUMOR	PER CENT CHANGE IN PENTOSE METHOD OF ANALYSIS	
	Mejbaum	Roe-Rice
#Ta 111* Ovarian papillary adenocarcinoma		+0.34
AFP 239* 2-acetylaminofluorene mammary tumor	+ 1.9	
Carcinoma† 1643	− 0.7	
" "	− 1.7	
Sarcoma‡ ACMCA II	− 2.9	
" "	− 3.5	
" "	− 0.7	
" "	+ 2.7	
" "	− 1.2	
" "	+17.7	+1.41

* Obtained from Dr. A. Symeonidis, Nat. Cancer Inst.

† Obtained from Dr. H. P. Morris, Nat. Cancer Inst. In its 47th transplant generation.

‡ Obtained from Dr. M. K. Barrett, Nat. Cancer Inst. In its 66th transplant generation.

TABLE 3

DATA SHOWING PRECISION OF PENTOSE METHODS USED

METHOD OF ANALYSIS	AV. PER CENT VARIATION OF DUPLICATE DETERMINATIONS	
	Unincubated samples	Incubated samples
Roe and Rice	0.90	0.80
Mejbaum	2.26	3.43
Brachet	5.67	5.97

precision. It is to be expected that the method of Brachet for pentose determination, which involves steam distillation, would not give results with the precision obtainable with the Roe and Rice and the Mejbaum methods, which determine pentose in blood filtrates directly. In the reports of Menkes, no mention is made of obtaining large apparent increases of pentose content following incubation. This situation was frequently encountered in our experiments when the Brachet method was employed. In using the method of Brachet, Menkes (5–8) and Steen (10, 11) followed a procedure of outstanding difficulty in which there are considerable possibilities of error.

SUMMARY

1. Studies upon the Menkes pentolysis test for malignancy, with three methods for the determination of pentoses in serum, have been made.

2. No evidence was obtained of the presence of a pentose-destroying factor in the sera of rats bearing tumors induced by injection of 20-methylcholanthrene and in the sera of rats with malignant transplanted tumors of four different types.

3. The Roe and Rice procedure for the determination of pentose was found to have greater precision than the methods of Mejbaum and of Brachet.

ACKNOWLEDGMENTS

Grateful appreciation is expressed to Dr. R. M. Choisser for the pathological diagnosis of the tumors produced by injection of 20-methylcholanthrene.

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Announcement

Inactive Compounds in Tumor Chemotherapy

A new service is being inaugurated in this issue of *Cancer Research*. A considerable number of investigators are now engaged in screening compounds for their effect on neoplastic growths. In order to minimize repetition of effort and to facilitate future compilation of data, *Cancer Research* will publish condensed information on compounds that have been shown to be *inactive* in such tests. The results will be published in an alphabetical tabular form similar to that employed in "An Index to Tumor Chemotherapy" by Helen M. Dyer (Federal Security Agency, Public Health Service, March, 1949), and the compounds will be listed in the annual index. Authors submitting such data for publication should make every effort to present all the information called for in the form indicated on the next page as accurately and concisely as possible. Readers should consult the authors for more detailed information on these compounds. Reprints of these tables will be available to the authors.

The information in this issue was supplied by investigators from the Pacific Coast area. We are indebted to this group for suggesting that such data be made generally available in as short a time as possible.

The first table of this new section appears on the following page.

Inactive Compounds in Tumor Chemotherapy

Investigator and agent	Source	Physical constant*	Tumor	Age of tumor or days after transplant	Host Species	No.	Dose†	No. treatments	Route	Vehicle
Windsor C. Cutting, Stanford Medical School										
5-Amino-6-methyluracil		m255–270	sp. mam. ca.		mouse, C3H	15	.5%	6–12 mos.	in food	ground lab. chow (Purina)
β-Furfuraldoxime	Eastman		"		"	19	.05%	"	"	"
Melamine	"		"		"	17	.5%	"	"	"
Morin	"		"		"	15	1	"	"	"
Rutin	USDA		"		"	"	.05%	"	"	"
B. L. Freedlander and Arthur Furst, Mt. Zion Hospital and University of San Francisco										
p-Aminobenzoylamide of 3-aminoquinoline		m155–156	S-37	0	mouse, Webster	10	8	8	oral	gum acacia
							"	"	ip	" "
p-Aminosalicylic acid hydrazone of acetaldehyde		m275d	"	"	"	"	6	7	"	" " +bicarbonate
1,4-Dicyanobenzene		s156	"	"	"	"	2	6	oral	peanut oil
							"	"	ip	" "
p-Dimethylaminophenyl-6-ethoxyquinaldyl carbinol		m153	S-180	"	"	"	8	9	in food	
p-Dimethylaminophenyl-6-methoxyquinaldyl ketimine		m222	"	"	"	"	7	"	"	"
p-Dimethylaminophenyl-quinaldyl ketone		m194–196	"	"	"	"	8	7	"	"
2-(*p*-Dimethylamino-styryl)-6-methoxy quinoline		m206	S-37	"	mouse, ABC	20	5–10	5	oral	peanut oil
Diphenyl phosphoric acid amide		m147–149	"	"	mouse, Webster	10	2	7	ip	gum acacia
2-Fluorenyl acrylic acid			"	"	"	"	.075	2	"	peanut oil
3-Nitro-4-hydroxyphenyl arsonic acid			"	"	"	"	4	7	"	gum acacia
2-(*p*-Nitrostyryl)-6-methoxy quinoline		m134	"	"	mouse, ABC	"	4	4	oral	peanut oil
1-Quinaldyl-2-hydroxy-3-trichloropropane		m146–147	BA	"	mouse, C3H	"	6–12	16	"	" "
"		"	S-180	"	mouse, Webster	"	3–6	8	"	" "
David M. Greenberg and E. M. Gal, Division of Biochemistry, School of Medicine, University of California										
DL-Allo-threonine		m250–254d	S-37	when palp.	mouse, A	10	2×75	10	ip	water
DL-α-Amino-α-methyl-butyric acid		m306	"	"	"	17	100	"	"	"
L-Carboxymethyl cysteine		m245d	"	"	"	10	16	"	"	"
DL-γ-Hydroxy-α-amino-butyric acid		m202d	"	"	"	20	10	"	"	"
Isopropylhomocysteine		m239d	"	"	"	10	25	"	"	"
DL-*p*-Nitrobenzoyl serine		m206	"	"	"	"	"	7	sc	"
DL-Phenylserine		m193–194d	Gardner lym-sar.	"	mouse, C3H	"	33	10	ip	"
Sodium octanoate			"	"	"	"	6.25	"	"	"
3-Tyrosine-HCl			"	"	"	"	35	"	"	"
Floyd C. Turner, Boulder Creek, Calif.										
3-Acetylpyridine	Farchan		sp.	varied	mouse, Webster	10	.55	100	ip	water
2-Aminopyrimidine	Eastman		"	"	"	"	7	49	"	"
Bromal (tribromacetaldehyde)		b174d	"	"	mouse, C3H	"	.03	40	"	"
o-Bromotoluene	Eastman		"	"	mouse, Webster	"	1.1	100	"	"
N-2-Chlorophenylphthalamic acid	U.S. Rubber Co.		"	"	"	"	10	25	"	"
Cupferron (ammonium nitrosophenylhydroxylamine		m163–164	tr. HP mel.	28	mouse, C	"	.5	58	"	"
Diacetyl monoxime	Eastman		tr. Cr-180 sar.	26	mouse, C3H	16	5	11	"	"
Dimethyl-*n*-propyl-carbinol	"		tr. HP mel.	28	mouse, C	10	1	52	"	"
Ethyl norvaline			tr. mca. sar.	15	mouse, DBA	"	2	32	"	"
Ethylene diamine		m85	tr. HP mel.	28	mouse, C	"	.5	33	"	"
Furan		m31.5	tr. mca. sar.	15	mouse, DBA	"	1.7	31	"	colloid in water
5-Nitrouracil	Eastman		"	"	"	"	3.75	17	"	"
Sodium phosphate (sec.)		m34.6	"	"	"	"	10	36	"	water
Uracil	Eastman		"	"	"	"	3.75	24	"	colloid in water
Darrell N. Ward and A. Clark Griffin, Stanford University										
1-Phenacyl-4(n-amyl)-pyridinium iodide		m175	azo dye ind. hepatoma	0–84 after dye started	rat, SD	59	3.5–120	1–24	in food or ip	10 per cent ethanol
"		"	"	"	rat, H	12	1–2	ad lib.	in food	
"		"	sp. leuk.		mouse, C58 black (MacDowell)	3	1	12 semi-weekly	ip	10 per cent ethanol
"		"	tr. leuk. #C 1534	1–7	mouse, DBA, line 2 (Jackson Mem. Lab.)	31	1%	1–10, daily	"	"

* m, b, s, and d = melting, boiling, sublimation, and decomposition points, respectively. Other abbreviations follow the style listed on page 10 of "An Index To Tumor Chemotherapy" by Helen M. Dyer.

† Unless otherwise specified all dosages are given in milligrams per animal.